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## Role of carotenoid cleavage dioxygenases in volatile emissions and insect resistance in Arabidopsis

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Graduate Program in Biology  
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science  
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**Role of carotenoid cleavage dioxygenases in volatile emissions and insect  
resistance in Arabidopsis**

(Thesis format: Monograph)

by

**Shailu Lakshminarayan**

Graduate Program in Biology

A thesis submitted in partial fulfillment  
of the requirements for the degree of  
Master of Science

The School of Graduate and Postdoctoral Studies  
The University of Western Ontario  
London, Ontario, Canada

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## Abstract

In *Arabidopsis thaliana*, carotenoid degradation by enzymatic oxidative cleavage is catalyzed by four carotenoid cleavage dioxygenase (CCD) and five nine-cis epoxycarotenoid dioxygenase (NCED) enzymes; this cleavage produces an array of terpenoid products that are collectively known as apocarotenoids, which include volatile and non-volatile compounds. Previous studies on CCD1 revealed that its overexpression in *A. thaliana* enhances  $\beta$ -ionone emission and reduces damage caused by flea beetles. In consideration of these findings, I generated *CCD4* and *CCD8* *Arabidopsis* overexpression lines. Further, I characterized these overexpression lines on the basis of their carotenoid content and their volatile profiles. Surprisingly, accumulation of key carotenoids in the leaves was observed in both CCD4 and CCD8 plants. In addition, GC-MS analysis indicated that the CCD4 and CCD8 lines showed variable changes in their volatile profiles relative to the control. Varying amounts of volatile apocarotenoids were observed in the CCD4 and CCD8 lines, and none were detected in the WT plants. In addition, there was a presence of other volatile compounds, including sesquiterpenes and monoterpenes, which were present in the transgenic lines; these volatile compounds have been found to be insect feeding deterrents. Further, insect bioassays were conducted using the crucifer flea beetles to determine if the CCD4 and CCD8 plants had enhanced deterrence of insect feeding. The CCD4 lines showed the highest insect feeding deterrence compared to the CCD8 and WT control lines suggesting that the volatile compounds released from these plants possibly had feeding deterrence towards this insect.

**Key Words:** CCD, NCED, carotenoids, volatile apocarotenoids, insect feeding  
deterrence, overexpression lines, crucifer flea beetles, insect bioassay

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## List of Abbreviations

ABA	abscisic acid
bLYC	lycopene $\beta$ -cyclase
bp	base pair
CCD	carotenoid cleavage dioxygenases
cDNA	complementary DNA
CH <sub>3</sub> COOK	potassium acetate
CTAB	cetyl-trimethylammonium bromide
DMADP	dimethylallyl diphosphate
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
eLYC	lycopene $\epsilon$ -cyclase
EMV	electron multiplier voltage
GC-MS	gas chromatography mass spectrometry
GGDP	geranyl geranyl diphosphate
GGDPS	geranyl geranyl diphosphate synthase
HIPVs	herbivore induced plant volatiles
HPLC	high performance liquid chromatography
IPDP	isopentyl diphosphate
KOH	potassium hydroxide
LUT1	$\epsilon$ -ring carotene hydroxylase
MEP	methylerythritol phosphate
MS	Murashige and Skoog

NaCl	sodium chloride
NaOH	sodium hydroxide
NCED	nine-cis-epoxycarotenoid dioxygenase
oligo dT	oligo deoxy-thymine
PCR	polymerase chain reaction
PDS	phytoene desaturase
PSY	phytoene synthase
RNA	ribonucleic acid
RT-PCR	reverse transcription-polymerase chain reaction
SDS	sodium dodecyl sulfate
SL	strigolactones
TAE	tris-acetate-EDTA
TBE	tris-borate-EDTA
v/v	volume per volume
VDE	violaxanthin deepoxidase
w/v	weight per volume
WT	wild type
ZDS	ζ-carotene desaturase
ZEP	zeaxanthin epoxidase

# **Chapter 1: Introduction**

## **1.1 Plant responses to insect feeding**

Almost all land ecosystems have been strongly influenced by interactions between plants and insects, which are the most diverse species on the planet (Whitney and Federle, 2013). Both insects and plants have the inherent ability to adjust their behaviors to suit their surrounding environment. In some cases, insect interactions with plants may be beneficial to the plant, for example insects assist with pollination and may protect the plant from feeding by more damaging pests. However, in many instances insect feeding on plants leads to severe damage resulting in major crop losses. To protect themselves from insects, plants have evolved complex direct and indirect defense mechanisms. Direct strategies include the presence of physical barriers such as thorns, hairs, wax, etc. (Kessler and Baldwin, 2002). Indirect mechanisms include the release of some secondary metabolites, which act as insect repellents (Dicke et al., 2009; Gatehouse, 2002). The focus of this thesis is the use of these secondary metabolites by plants to defend themselves against insect herbivory.

Plants produce a diverse range of volatile compounds, and some of them are secondary metabolites (Pichersky and Gang, 2000). The mixture of volatile compounds emitted from plants may include terpenoids, phenylpropanoids/benzenoids, fatty acid derivatives, and amino acid derivatives (Dudareva and Pichersky, 2000). These volatile compounds are released from leaves, flowers, and fruits into the atmosphere and the volatiles from the roots are released into the soil (Dudareva and Pichersky, 2008). The main functions of plant-derived air-borne volatiles are defense against herbivores and

pathogens, to attract pollinators, seed dispersers, and beneficial animals and organisms, and to act as signals in plant-plant interactions (Dudareva and Pichersky, 2008). These volatiles can serve in promoting or deterring interactions between plants and insect herbivores. For example, without herbivore damage, wheat seedling volatiles attract aphids, whereas the odors released from wheat seedlings infested with a high density of aphids repels other aphids (Quiroz et al., 1997). Volatile compounds released in response to insect feeding can serve as chemical signals for herbivores (Pare and Tumlinson, 1997b). In tritrophic interactions, plants can defend themselves against herbivorous feeding by producing volatiles that attract natural enemies of the herbivores (Birkett et al., 2000; Dicke et al., 1990). Another example of this type of interaction is that of the caterpillar crucifer pest *Pieris rapae*. On feeding by these caterpillars, volatiles are released from the plant that attracts the parasitoid wasp, *Coesia rubecula*. These wasps are predators of the *P. rapae* caterpillars (van Poecke and Dicke, 2002; Van Poecke et al., 2001). In a contrary example, volatiles from host plants enhance the effect of contact stimulants of lepidoptera, thus increasing the landing rates and laying of eggs (oviposition) relative to non-host plants (Feeny et al., 1989). Upon attack by insects, plants emit over 200 different volatile organic compounds (VOCs) (Dicke and van Loon, 2000). These emitted volatiles can directly intoxicate, repel or deter herbivorous insects (Bernasconi et al., 1998; De Moraes et al., 2001; Kessler and Baldwin, 2001). Furthermore, studies have reported that Arabidopsis and maize plants overexpressing terpene synthases attract enemies of herbivores under laboratory conditions (Kappers et al., 2005; Schnee et al., 2006); in the field, the plants that constitutively produce  $\beta$ -

caryophyllene attracted nematodes, which as a result reduced the survival of the western corn rootworm *Diabrotica virgifera* (Degenhardt et al., 2009).

Undamaged plants maintain a baseline level of volatile metabolites that are usually released from the surface of leaves or from accumulated storage sites in the leaf (Pare and Tumlinson, 1997a). These chemicals consist of a blend of monoterpenes, sesquiterpenes, and aromatic compounds (Pare and Tumlinson, 1997b). There is a similarity in the structure of volatile compounds that are emitted from insect-damaged leaves and leaf regions in proximity to damaged areas; furthermore, the chemical products are produced in response to a wide range of insect parasitoids and predators (Pare and Tumlinson, 1999). Although the individual components of herbivore-induced volatile blends belong to various chemical classes, isoprenoids are the dominant composition of many of these blends (Arimura et al., 2004; Boland et al., 1992; Dudareva et al., 2004; Pare and Tumlinson, 1997a).

## **1.2 Role of volatiles in plant-insect interactions**

Crop damage due to severe insect feeding has been a concern in agriculture. This problem has prompted plant biologists to study the mechanism of plant insect interactions and manipulate these interactions in a way that would make plants more resistant to insect feeding damage. Plant volatiles often referred to as herbivore induced plant volatiles (HIPVs), act as repellents to herbivore pests and attractants for biological control agents (parasitoids and predators) (Hammack, 2001; Khan et al., 2008; Nojima et al., 2003). The role of HIPVs in repelling herbivores from plants was supported by evidence from feeding deterrence trials. For example, the red-legged earth mite *Halotydeus destructor* Tucker (Acari: Pentheleidae) fed less on *Trifolium glanduliferum*



extracts that had high levels of  $\beta$ -ionones and other terpenes (Wang et al., 2005). In addition, methyl salicylate inhibited feeding and egg-laying activity by western flower thrips when applied to the leaf surface of bean and cucumber (Koschier et al., 2007). HIPVs have been demonstrated to play a role as attractants of predators such as *Phytoseiulus persimilis*, *Geocoris pallens* and green lacewing (De Boer and Dicke, 2004; James, 2003; Kessler and Baldwin, 2001). Kessler and Baldwin (2001) reported that herbivorous damage in *Nicotiana attenuata* plants caused by tobacco flea beetle *Epitrix hirtipennis* (Coleoptera, Chrysomelidae) and other leaf-feeding herbivores led to the emission of terpenoid compounds linalool and cis-bergamotene, and green leaf volatile cis-3-hexen-1-ol. This in turn increased egg predation rates by a generalist predator and decreased lepidopteran oviposition rates, consequently, resulting in reduction of the herbivores number by more than 90% via the release of volatiles. Also, volatile compounds emitted from whitefly-infested bean leaves were shown to significantly increase flight orientation and landing by the whitefly parasitoid, *Encarsia formosa*, in wind tunnel bioassays (Birkett et al., 2003). Volatiles are also used by herbivores to locate their host plant or a trap crop (Badenes-Perez et al., 2004), and feeding causes the plant to produce a different set of volatiles that can attract more herbivores of the same species (Addesso and McAuslane, 2009). Among plant volatiles, terpenoids have been well documented to act as toxins, feeding deterrents, or oviposition deterrents to a range of insects (De Moraes et al., 2001; Gershenzon and Croteau, 1991; Litvak and Monson, 1998; Raffa et al., 1985; Wei et al., 2004a).

### 1.3 Carotenoids and Apocarotenoids

Among the hundreds of volatile compounds released from plants, terpenoids constitute a diverse class of naturally occurring organic chemicals, and are known for their feeding deterrent properties (De Moraes et al., 2001; Gershenzon and Croteau, 1991; Litvak and Monson, 1998; Raffa et al., 1985; Wei et al., 2004a; Wei et al., 2004b). These compounds are derived from the isoprenoid pathway and are known for their flavor and aroma, and they play a crucial role in plant physiology (Aharoni et al., 2005). They are synthesized in various parts of the plant cell, such as in the plastids and cytoplasm, and are stored in specialized secretory structures. These structures are generally located in regions that would most likely help defend the various organs, e.g., trichomes on the surface of leaves, pockets near the epidermis of primary stems, in fruit, etc. (Gershenzon and Croteau, 1991). Terpenoids are derived from five-carbon isoprene units assembled and modified in many ways. They are classified into different categories depending on the number of repeating 5-carbon units that they contain. These include C<sub>10</sub> (mono-), C<sub>15</sub> (sesqui-), C<sub>20</sub> (di-), C<sub>30</sub> (tri-), C<sub>40</sub> (tetra-), and C>40 (poly-) terpenoids (Langenheim, 1994).

Carotenoids are one of the most studied classes of terpenoids. Carotenoids are C<sub>40</sub> isoprenoids (Lu and Li, 2008) that play critical roles not only in plant defense, but in plant growth and development, in addition to their many economic and health benefits (Carvajal-Lérida et al., 2012). They are precursors of vitamin A, and some carotenoids are used as food colorants in the food and cosmetics industries (Beatty et al., 2004; Umeno et al., 2005). Carotenoids are critical components of the photosynthetic machinery, and play a role in protecting the plant from photooxidative damage (Howitt

and Pogson, 2006). Carotenoids are multifunctional compounds and serve as structural components of the light harvesting complexes, accessory pigments for light harvesting, and components of photoprotection and scavengers of singlet oxygen (DellaPenna, 2004). They provide the yellow, orange, and red colors to fruits and flowers. Carotenoids are synthesized in nearly all types of plastids but they accumulate in large amounts in chloroplasts and chromoplasts (Howitt and Pogson, 2006). Distinct regulatory mechanisms of carotenoid biosynthesis are active in green tissues, flowers, and fruits (Lu and Li, 2008). The most abundant carotenoids in the leaves include lutein,  $\beta$ -carotene and violaxanthin. Lutein is a dihydroxy derivative of  $\alpha$ -carotene. It is the most abundant carotenoid in the photosynthetic plant tissues and plays an important role in the structure and function of the light harvesting complex II (DellaPenna and Pogson, 2006). The presence of violaxanthin and other carotenoids confer the yellow coloration to the chloroplast membranes (Markwell et al., 1992). Most chlorophyll carotenoids are located along with chlorophyll in the photosynthetic (thylakoid) membranes (Demmig-Adams et al., 1996).  $\beta$ -Carotene is usually present in abundance in the photosystem reaction centres, while the xanthophylls, such as violaxanthin, are abundant in the light-harvesting complexes (Davison et al., 2002; Pogson et al., 1998).

Carotenoids are in constant turnover; i.e. biosynthesis and catabolism, and oxidative cleavage of carotenoids produces terpenoid compounds known as apocarotenoids (Wahlberg and Eklund, 1998). Apocarotenoids include biologically active compounds such as various plant hormones, including abscisic acid (ABA) and strigolactones (SL) (McCarty, 1995), and volatile flavor and fragrance compounds (Mendes-Pinto, 2009). Some of the commonly known volatile apocarotenoids include  $\beta$ -

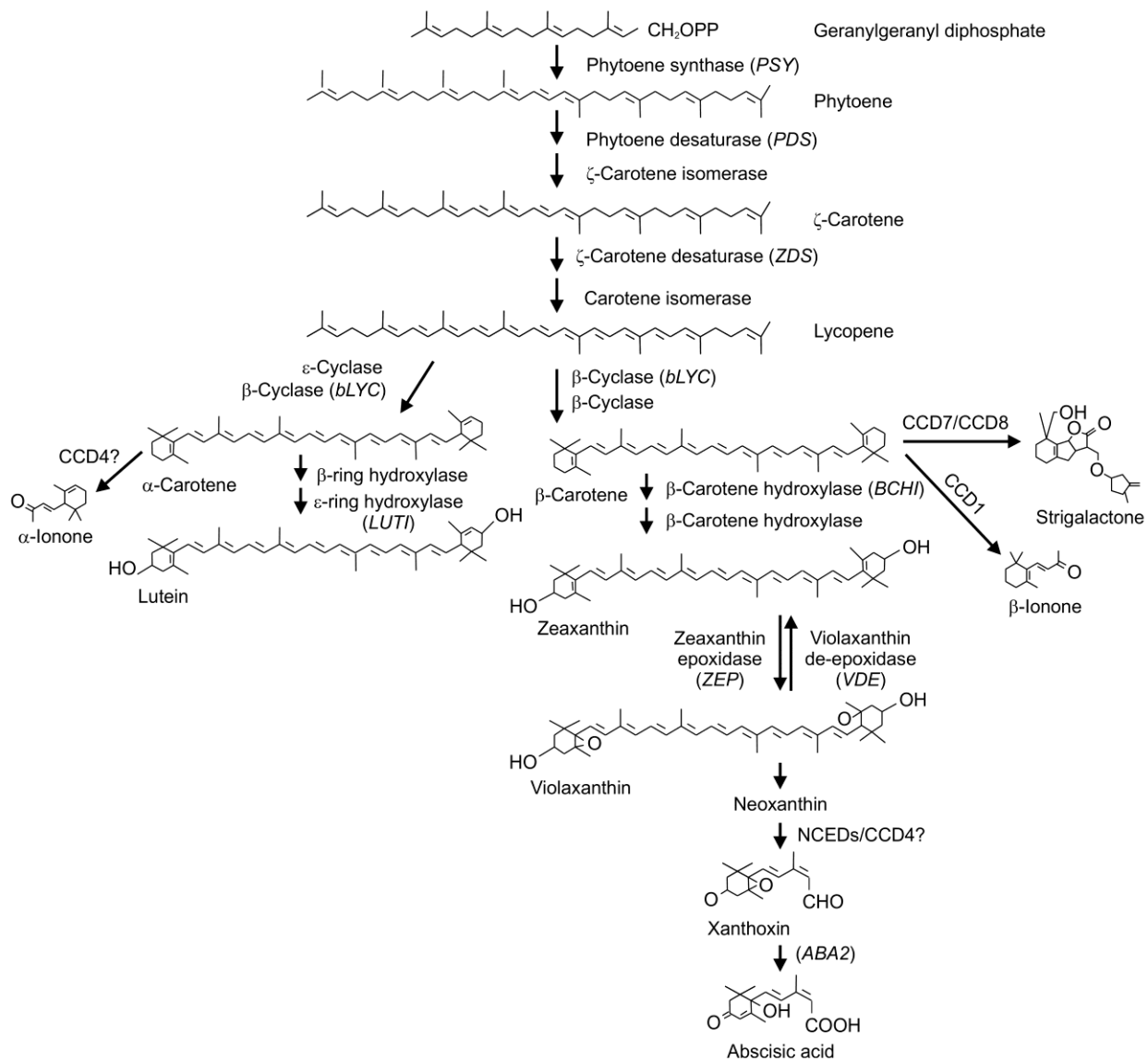
ionone,  $\beta$ -cyclocitral, theasporone,  $\beta$ -damascenone, and  $\alpha$ -damascenone.

Apocarotenoids are generated when double bonds in the carotenoid backbone are cleaved by molecular oxygen forming an aldehyde and ketone from each substrate at the site of cleavage. Carotenoids can be cleaved at any of their double bonds resulting in a diverse set of apocarotenoids (Vogel et al., 2008). Although apocarotenoid formation may occur through non-specific oxidation, the biologically active forms that possess regulatory functions are formed as a result of site-specific oxidative cleavage (Vogel et al., 2008).

### **1.3.1 Carotenoid biosynthesis and catabolism in plants**

The carotenoid biosynthesis pathway begins with the synthesis of the five-carbon building block, isopentyl diphosphate (IPDP) and its allylic isomer, dimethylallyl diphosphate (DMADP). IPDP and DMADP are derived from the methylerythritol phosphate (MEP) pathway (Eisenreich et al., 2001; Hunter, 2007). Three molecules of IPDP are combined with DMADP by geranyl geranyl diphosphate (GGDP) synthase (GGDPS) to produce a 20-carbon molecule, GGDP, which is the precursor of the carotenoid biosynthesis pathway (Figure 1). The first committed step in the carotenoid biosynthesis pathway is the condensation of two GGDP molecules by phytoene synthase (PSY) to produce a 40-carbon molecule, phytoene. This is a rate-limiting step in the carotenoid pathway (Lu and Li, 2008). The next step involves the desaturation of phytoene into red colored lycopene by phytoene desaturase (PDS) and  $\zeta$ -carotene desaturase (ZDS). Lycopene is the critical branching point in the pathway (Cazzonelli and Pogson, 2010). It is cyclized to yield either  $\alpha$ -carotene by lycopene  $\epsilon$ -cyclase (eLYC) and lycopene  $\beta$ -cyclase (bLYC) or  $\beta$ -carotene by bLYC alone.  $\alpha$ -carotene and  $\beta$ -carotene

are hydroxylated to produce lutein and zeaxanthin, respectively. These hydroxylation reactions are catalyzed by the  $\beta$ -ring carotene hydroxylase and the  $\epsilon$ -ring carotene hydroxylase (LUT1) (Galpaz et al., 2006; Kim and DellaPenna, 2006; Tian et al., 2004). Lutein is one of the most abundant carotenoids, and is present in the leaf tissues of most plants. Epoxidation of zeaxanthin by zeaxanthin epoxidase (ZEP) produces violaxanthin. This reaction is reversed by violaxanthin deepoxidase (VDE) to give rise to the xanthophyll cycle, which helps plants acclimatize to high light stress. Violaxanthin is further converted to neoxanthin by neoxanthin synthase (NSY). The formation of neoxanthin represents the last step in the carotenoid biosynthesis pathway (Lu and Li, 2008). The end products of the pathway can be catabolized to produce apocarotenoids. The carotenoid cleavage dioxygenase (CCD) enzymes target various non-specific carotenoids in the pathway, whereas the nine-cis-epoxycarotenoid dioxygenases (NCEDs) are predominantly responsible for cleaving violaxanthin and neoxanthin to produce xanthoxin, the direct substrate for ABA synthesis. The enzyme ABA2 uses xanthoxin as a substrate in the ABA conversion step (Figure 1).



**Figure 1: The carotenoid biosynthesis pathway in plants.** The pathway illustrates the first committed step in the carotenoid biosynthesis pathway, which involves the conversion of GGDP to phytoene. The pathway branches out at lycopene. This pathway indicates the involvement of the CCD and NCED enzymes at the various steps. ? – indicates that the mechanism of the CCD enzyme in the particular step is not clear. CCD – carotenoid cleavage dioxygenases; NCED – nine-cis-epoxycarotenoid dioxygenases. The genes that encode for the key enzymes involved in the carotenoid biosynthesis pathway are indicated in brackets.

### 1.3.2 Regulation of carotenoid biosynthesis

Since carotenoids play a key role in the photosynthetic machinery, the regulation of carotenoid biosynthesis in green tissues of plants occurs in a coordinated manner with the other cellular processes involved in photosynthesis (Lu and Li, 2008). Typically there are four carotenoids that accumulate in the chloroplasts of higher plants: lutein,  $\beta$ -carotene, violaxanthin, and neoxanthin (DellaPenna and Pogson, 2006). Although chloroplast metabolic processes are generally governed by light-mediated regulation of the relevant genes, this is not necessarily the case for carotenoid biosynthetic genes. Although certain carotenoid biosynthetic genes such as *PSY* are regulated by light through the phytochrome mediated process (von Lintig et al., 1997; Woitsch and Romer, 2003), the transcript levels of the carotenoid genes together are not light dependent (Fraser and Bramley, 2004). During the development of fruit color and fruit ripening, the transcriptional regulation of carotenoid gene expression appears to be the key machinery by which the biosynthesis and accumulation of specific carotenoids are regulated (Lu and Li, 2008). There have been studies that clearly pointed out that oxidative cleavage of carotenoids is induced by environmental stresses (Han et al., 2004). The circadian rhythm has also been shown to affect carotenoid catabolism (Simkin et al., 2004b).

### 1.3.3 Carotenoid cleavage dioxygenases

The CCDs form a family of enzymes that are involved in the oxidative cleavage of carotenoids and apocarotenoids. The first described member of the carotenoid cleaving enzymes was the VIVIPAROUS14 (VP14), which was first identified in maize (*Zea mays*). VP14 is a NCED involved in the asymmetrical cleavage of the 11, 12 (11', 12') double bonds of neoxanthin and violaxanthin leading to the formation of abscisic acid



(Schwartz et al., 1997b; Tan et al., 1997). Studies on VP14 opened doors to the discovery of related enzymes in other different plant species, and a number of *CCD* and *NCED* genes have been found in a many plant species (Qin and Zeevaart, 2002), including tomato (Burbidge et al., 1999), bean (Qin and Zeevaart, 1999), *Arabidopsis* (Iuchi et al., 2001; Tan et al., 2003), avocado (Chernys and Zeevaart, 2000), and orange (Rodrigo et al., 2006), to name a few.

In *Arabidopsis thaliana*, there exist nine *CCD* and *NCED* genes, five of which are involved in abscisic acid biosynthesis and are classified as *NCED* genes (*NCED2*, *NCED3*, *NCED5*, *NCED6*, and *NCED9*), and the remaining four are classified as *CCD* genes (*CCD1*, *CCD4*, *CCD7*, and *CCD8*) (Iuchi et al., 2001; Tan et al., 2003). *CCD* and *NCED* enzymes differ on the basis of their preferred substrate and presumed mechanism of catalysis (AuldrIDGE et al., 2006b). The *CCDs* and *NCEDs* in higher plants contribute to various physiological processes, including the regulation of lateral shoot buds (Alder et al., 2008) and plastid development (Naested et al., 2004). The members of the *CCD* and *NCED* family share certain characteristics: 1) they require a  $\text{Fe}^{+2}$  for catalytic activity (Kiefer et al., 2001; Redmond et al., 2001; Schwartz et al., 1997a); 2) they contain four conserved histidines that are believed to coordinate with the iron binding; and 3) they contain a conserved peptide sequence at their carboxyl terminus that constitutes a signature sequence for the family (AuldrIDGE et al., 2006b). *CCD1* enzyme cleaves a broad range of carotenoids, such as lycopene,  $\beta$ -carotene,  $\delta$ -carotene, zeaxanthin, violaxanthin, and neoxanthin, to generate aldehydes and ketones that are volatile aroma compounds (AuldrIDGE et al., 2006b; Schwartz et al., 2001; Simkin et al., 2004a). In petunia flowers *CCD1* controls the emission of  $\beta$ -ionone (Simkin et al., 2004b) and in

tomato CCD1 generates flavor volatiles such as geranylacetone, pseudoionone, and  $\beta$ -ionone (Simkin et al., 2004a). Some of the volatile apocarotenoids that are commonly known to be produced by CCD1 include  $\beta$ -ionone,  $\alpha$ -ionone, 3-hydroxy- $\beta$ -ionone, pseudoionone, geranylacetone, and 6-methyl-5-hepten-2-one (Vogel et al., 2008). CCD4 catalyzes the cleavage of carotenoids forming aroma compounds, such as  $\beta$ -ionone (Bouvier et al., 2003; Huang et al., 2009b; Ohmiya et al., 2006) and CCD7 and CCD8 catalyze the sequential cleavage of carotenoids to form SL, the hormone involved in the inhibition of shoot branching (Auldridge et al., 2006b; Domagalska and Leyser, 2011; Waters et al., 2012).

The metabolic turnover of carotenoids by CCDs and NCEDs not only produces important signaling and accessory apocarotenoid molecules, but helps to maintain a steady-state level of carotenoids in plants.

#### **1.4 Production of apocarotenoid volatiles by CCDs and NCEDs, and role of volatiles in plant insect interactions in plants**

Ever since the cloning of the *Arabidopsis* *CCD* and *NCED* genes, many studies have been conducted to characterize the enzymes in terms of their substrate specificities and functions in the carotenoid biosynthesis pathway. Although the CCD and NCED enzymes exhibit specificity for the double bond at which they cleave, some of them are less specific in terms of their substrate choices (Auldridge et al., 2006a).

The first member of the CCD1 subfamily was identified from *Arabidopsis* (Schwartz et al., 2001) and was found to act as a dioxygenase. Sequence homology helped in characterizing and identifying the orthologs of this gene in several plant species

such as tomato, grape, maize, melon, petunia (Bouvier et al., 2003; Mathieu et al., 2005; Simkin et al., 2004a). A study by Vogel et al. (2008) suggested that CCD1 has broad substrate specificity and cleaves numerous cyclic and linear all-trans-carotenoids, such as  $\zeta$ -carotene, lycopene,  $\beta$ -carotene,  $\delta$ -carotene, and zeaxanthin at the 9, 10 and 9', 10' double bonds into C<sub>13</sub> ketones and two variable end-group derived C<sub>13</sub> aldehydes. CCD1 activity produces a variety of C<sub>13</sub> compounds, including  $\beta$ -ionophores,  $\alpha$ -ionones, pseudoionone, and geranylacetone (Simkin et al., 2004b; Vogel et al., 2008).

The biological function of *CCD1* loss-of-function tomato fruits showed decreased levels of  $\beta$ -ionone, pseudoionone, and geranylacetone, and this suggested that CCD1 plays a role in the production of  $\beta$ -ionone (Simkin et al., 2004a). In addition, experiments on the seeds of *Arabidopsis ccd1* mutants suggested that the levels of carotenoid were elevated, a finding that implicated CCD1 in carotenoid catabolism (Auldrige et al., 2006a). Silencing of the tomato *CCD1* (*LeCCD1A* and *LeCCD1B*) resulted in a decrease in fruit volatile apocarotenoids, such as  $\beta$ -ionone and geranylacetone, thus suggesting a link between CCD1 and apocarotenoid production *in vivo* (Simkin et al., 2004a).

Although it has been found that *CCD1* expression is elevated during fruit development in tomatoes and grapes, it does not necessarily reflect the levels of volatile apocarotenoids being emitted (Mathieu et al., 2005; Simkin et al., 2004a). The lag in CCD1 gene expression and apocarotenoid production could be due to differences in sub cellular localizations and availability of substrates (Bouvier et al., 2003). Other studies have found that the modification of *CCD1* expression could increase the carotenoid content in plant tissues (Romer et al., 2000; Rosati et al., 2000). Several studies have clearly linked the emission of volatile apocarotenoids, such as  $\beta$ -ionone, with the overexpression of

*CCD1* in *Arabidopsis* (Wei et al., 2011). It has been reported that *CCD1* apocarotenoid products have antimicrobial activities (Fester et al., 1999). Therefore, expression of *CCD1* in all tissues may be important for plant defenses (Auldridge et al., 2006a). These studies provided clear evidence that genetic manipulations of *CCD1* can alter the carotenoid and apocarotenoid content of the plant.

The other *CCD* genes have also been characterized. For example, *CCD4* is found to play a role in maintaining balanced levels of carotenoids that contribute to the pigmentation of petals; RNAi experiments performed by Ohmiya et al. (2006) showed that suppressing *CCD4* in the *Chrysanthemum* white flowered cultivars resulted in the petal color turning yellow. This suggested that Cm*CCD4* contributed to the white color formation in the petals by cleaving carotenoids into colorless compounds (Ohmiya et al., 2006). Campbell et al. (2010), using potato tubers, showed that down-regulation of the potato *CCD4* using RNAi resulted in an increase in the total tuber carotenoid content (Campbell et al., 2010). Other findings of this study indicated that down-regulation of *CCD4* in potato tubers led to elevated levels of the carotenoid violaxanthin (Campbell et al., 2010). This suggested that violaxanthin could be one of the main substrates for the *CCD4* enzyme in potato. However, Huang et al. (2009b) showed that  $\beta$ -carotene is one of the main substrates for the *CCD4* enzyme of five different plant species, namely, *Chrysanthemum* (*Chrysanthemum morifolium*), apple (*Malus domestica*), rose (*Rosa damascene*), osmanthus (*Osmanthus fragrans*), and *Arabidopsis* (Huang et al., 2009b).

Other groups have shown that *CCD7* and *CCD8* are responsible for the synthesis of the novel apocarotenoid hormone, SL, which controls leaf and lateral shoot growth (Booker et al., 2004; Sorefan et al., 2003). Further, work on *CCD7* showed that the

enzyme cleaves multiple carotenoids including lycopene,  $\beta$ -carotene, and zeaxanthin (Booker et al., 2004). When the activity of recombinant CCD8 was tested using carotenoid substrates, after expressing the full-length CCD8 with an N-terminal GST (glutathione-S-transferase) fusion in *Escherichia coli* strains engineered to accumulate lycopene,  $\beta$ -carotene or zeaxanthin, the accumulation of these carotenoids was significantly reduced when CCD8 expression was induced (Cunningham et al., 1996; Sun et al., 1996). Auldridge et al. (2006a) showed that CCD7 has a broad substrate specificity and that it uses both linear and cyclized carotenoid substrates, cleaving them at the 9, 10 (9', 10') double bonds to generate two aldehyde molecules (Booker et al., 2004). It has also been shown that CCD7 and CCD8 cleave carotenoids in a sequential manner, whereby CCD7 cleaves  $\beta$ -carotene asymmetrically, generating a C<sub>13</sub> ( $\beta$ -ionone) and a C<sub>27</sub> aldehyde (10'-apo-  $\beta$ -carotenal), and then CCD8 catabolizes the C<sub>27</sub> aldehyde to generate a C<sub>18</sub> aldehyde and C<sub>9</sub> dialdehyde (Schwartz et al., 2004).

Among the five NCED enzymes, NCED3 plays a major role in the regulation of ABA synthesis in response to stress in the leaves, while the remaining four NCEDs are mainly regulated during plant development (Iuchi et al., 2001; Ruggiero et al., 2004). The NCEDs are predominantly known to cleave the 11, 12 (11', 12') double bonds of carotenoid molecules, specifically xanthophylls, such as neoxanthin or violaxanthin (Schwartz et al., 1997a). NCEDs are primarily located in sites where ABA synthesis occurs, such as the lateral root initials, organ primordial and reproductive structures (Tan et al., 2003).

Although there have been functional studies on CCD enzymes expressed in *E. coli* to determine their enzymatic activities and substrate preferences, very few studies

have focused on measuring volatiles generated as a result of CCD and NCED expression due to the difficulty of performing these experiments *in vitro*. A ground breaking study, conducted by Wei et al. (2011), showed that transgenic *Arabidopsis* overexpressing *CCD1* exhibited enhanced levels of  $\beta$ -ionone, a volatile apocarotenoid produced as a result of CCD1 dioxygenase activity. Moreover, when transgenic plants with elevated levels of  $\beta$ -ionone were tested for their interaction with *Phyllotreta cruciferae* (crucifer flea beetles), feeding damage was reduced in the transgenic plants compared to WT plants. This suggested that the volatile apocarotenoids deterred the insects from feeding on these plants.

## 1.5 Crucifer flea beetles

The crucifer flea beetle, *P. cruciferae* (Goeze), is a common pest of cruciferous crops in Europe (Dirimanov and Angelova, 1964; Dobson, 1956; Newton, 1928) and India (Batra, 1969; Gupta, 1945; Varma, 1961). In Canada, this insect was first reported in 1923 in British Columbia, and the beetles started eastward migration in the 1930s, causing immense damage to *Brassica napus* (canola) and several other cruciferous crops on the Canadian prairies (Kinoshita et al., 1979). These beetles are known to attack plants belonging to the mustard family (Brassicaceae).

Although the life history of the flea beetles in Europe, India, and the United States are reasonably well understood, the life cycle of these beetles in Southwestern Ontario is not very clear; this has made it difficult to develop effective control measures against flea beetles (Kinoshita et al., 1979). The adult flea beetles are known to feed on the surface of leaves, stems, and seed pods, producing pits, and damaged leaves and cotyledons have a distinctive shot hole appearance. Heavy infestations may severely damage cotyledons,

petioles, and stems. The larvae feed on root hairs and roots, thus affecting the growth and development of crops, and further, reducing crop yield. Crucifer flea beetles have a single generation per year. In the spring, the overwintered adults emerge and feed on crucifer seedlings. In the fall, the offspring of the overwintered adults emerge and they usually feed on crucifer leaves, stems, and seed pods. In late August and September, the adults move into leaf litter and debris to overwinter (Burgess, 1977).

Pest management of crucifer flea beetles has been quite challenging due to the fact that the beetles attack and quickly injure or destroy seedlings shortly after the beetles' emergence. Various strategies that are being used include biological control and chemical control. Chemical control includes the use of insecticides that can be sprayed directly on the crops (Perring et al., 1999). Biological control measures include the use of predators and parasites, such as the lacewing larvae (*Chrysopa carnea*), big-eyed bugs (*Geocoris bullatus*), and braconid wasps (*Microctonus vittatae*), to regulate the flea beetle population (Burgess, 1977). Unfortunately, since the flea beetles emerge in large numbers during a relatively short period of time, they tend to overwhelm the plants. Therefore, it is necessary to develop a method to prevent the population of flea beetles from feeding on crucifer crops. An effective method would be to develop crops that are naturally resistant to insect feeding damage. This can be achieved through the use of genetic manipulations in the plant.

## **1.6 Hypothesis**

Volatile apocarotenoids released from plants affect the feeding behavior of insects. Furthermore, *CCD* genes are known to be associated with the production of apocarotenoids as a result of oxidative cleavage of carotenoids in the carotenoid

biosynthesis pathway. Consequently, I hypothesize that overexpression of the *CCD* and *NCED* genes, namely, *CCD4*, *CCD7*, *CCD8*, *NCED2*, *NCED3*, *NCED5*, *NCED6*, and *NCED9* in *Arabidopsis* will result in enhanced production of volatile apocarotenoids in the plants, thus reducing the feeding damage by crucifer flea beetles.

## 1.7 Thesis objectives

The oxidative cleavage of carotenoid compounds via the action of *CCD* and *NCED* enzymes results in emission of volatile and non-volatile apocarotenoids. Volatile apocarotenoids, such as  $\beta$ -ionone,  $\alpha$ -ionone,  $\beta$ -damascone, pseudoionone,  $\beta$ -cyclocitral, geranylacetone, which contribute to the flavour of fruits and scents of flowers, also affect insect feeding on the plant. Previous studies showed that overexpression of *CCD1* in *Arabidopsis* resulted in plants that exhibited significantly enhanced  $\beta$ -ionone emissions, and improved resistance to the beetle feeding. Therefore, this thesis focuses on characterization of the remaining *CCD* and *NCED* genes. Specifically, this research will investigate the effects of overexpressing some of these genes on volatile emissions and insect feeding. The objectives of the present research are:

- 1) Constitutively overexpress three *CCD* and five *NCED* genes in *Arabidopsis thaliana* and perform molecular characterization of transgenic plants.
- 2) Perform chemical characterization on the transgenic *Arabidopsis* plants to determine the effect of overexpressing the *CCD* and *NCED* genes on carotenoid content using HPLC, and on volatile apocarotenoids using GC-MS.
- 3) Perform insect feeding bioassays using the crucifer flea beetle to determine the effect of *CCD* and *NCED* gene overexpression on insect feeding damage.



## Chapter 2: Materials and Methods

### 2.1 Plant materials and growth conditions

*Arabidopsis thaliana* (Arabidopsis) Columbia-0 (Col-0) plants were grown in pots containing Pro-mix-BX soil. The seeds were sown onto pre-moistened soil in 4-inch plastic pots, and the surface of the soil was given adequate moisture. Once the trays containing the pots were covered with a plastic dome to maintain humidity, they were placed at 4 °C in the dark for 3-4 days to stratify the seeds. Subsequently, the trays were transferred to a growth room. The growth room was maintained at a controlled environment of 20 °C  $\pm$  3 °C with a photoperiod of 16 h, light intensity of 95–130  $\mu\text{mol}/\text{m}^2/\text{s}$ , and humidity set at 70%. The plastic domes were removed one week after the seeds germinated. The seedlings were watered three times a week, on every alternate day. Once the plants were two weeks old, they were supplemented with All-purpose Fertilizer 20-20-20 (1 gm/L), which was added to the soil on a bi-weekly basis. Three- and four-week-old plants were used for most of the experiments, and six-week-old plants with flowers were used for the volatile collection and insect bioassay experiments.

### 2.2 Isolation of cDNAs for *NCED2*, *NCED3*, *CCD4*, *NCED5*, *NCED6*, and *CCD8* genes

Organ-specific gene expression data were extracted from the Arabidopsis eFP browser at The Arabidopsis Information Resource (TAIR), which is based on Affymetrix ATH1 GeneChip data (<http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi>). Differential expression levels of the above six genes in Arabidopsis are listed in Table 1. Based on

these data, total RNA was extracted from late flowers, seed cotyledon (green siliques), and cauline leaves of three-week-old *Arabidopsis* plants. RNA extraction was performed on various tissues based on the differential expression levels in the various parts of the *Arabidopsis* plants (Table 1). RNA extraction from the tissue samples (100 mg) was performed using the RNeasy Mini kit (QIAGEN). The quality of RNA was confirmed using gel electrophoresis, and the spectrophotometer (NanoVue, GE Healthcare) readings were measured to confirm that the  $A_{260/280}$  ratio was in the range of 1.8–2.0. DNase treatment was performed after RNA extraction to remove DNA contamination from the samples. The TURBO DNase (Ambion Cat. # AM2238) enzyme was used for this treatment, according to manufacturer's instructions. First strand cDNA synthesis was performed using 1  $\mu$ g of total RNA using oligo dT primers (Superscript III Reverse Transcriptase kit; Life Technologies). Full-length cDNAs were amplified using gene specific primers (Table 2) that were designed to bind within the UTR regions of the genes (Table 2). High fidelity Taq polymerase (Platinum® Taq DNA Polymerase High Fidelity; Life Technologies) was used for gene amplification. Relevant primers (Table 2) were designed using the available gene sequence information ([www.arabidopsis.org](http://www.arabidopsis.org)). Primers were designed to include the leader sequence (5'-CACCC-3') suited for the recombination based Gateway® cloning technology (Life Technologies, USA). The primers were designed using the DNASTAR Lasergene Core Suite software (version 10; Madison, USA), and were further optimized using gradient PCR to determine the optimum annealing temperature based on the primer melting temperature ( $T_m$ ). The amplified cDNA was confirmed on a 1 % (w/v) agarose gel in 0.5× TBE buffer (1 M Tris, 900 mM

Borate, 25 mM EDTA, pH 8.3) to confirm the size of the products. The PCR products were confirmed by sequencing.

**Table 1: Tissue-specific expression levels of *CCD* and *NCED* genes in Arabidopsis.**

On the basis of the eFP browser (<http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi>) each *CCD* and *NCED* gene was differentially expressed in the different tissues of the plant.

Gene Name	Accession number	Plant part exhibiting high expression	Size of coding region of the gene (bp)
<i>CCD1</i>	AT3G63520	Cauline leaf	1617
<i>CCD4</i>	AT4G19170	Cauline leaf	1788
<i>CCD7</i>	AT2G44990	Dry seed	1890
<i>CCD8</i>	AT4G32810	Flower stage	1713
<i>NCED2</i>	AT4G18350	Late flower stage, early seed stage	1752
<i>NCED3</i>	AT3G14440	Flower stage sepals	1800
<i>NCED5</i>	AT1G30100	Seed stage (green cotyledon)	1770
<i>NCED6</i>	AT3G24220	Seed stage (green cotyledon)	1734
<i>NCED9</i>	AT1G78390	Seed stage/silique	1974

**Table 2: List of primers used in cloning, sequencing and qRT-PCR**

<b>Genes</b>	<b>Primer names</b>	<b>Primer sequences (5' - 3')</b>	<b>Primer use</b>	<b>Melting temperature (T<sub>m</sub>) in °C</b>
<i>CCD4</i>	CCD4 For	CACCATGGACTCTGTTTCTTCTTCCTT	Gene specific	62.1
	CCD4 Rev	TAAAGCTTATTAAGGTCACCTTCCTTGACAA	Gene specific	58.6
	CCD4-Int-For	TCACGCCATAAAAAATCCACAACG	Gene specific sequencing	58.5
	CCD4-Int-Rev	CGTGAATGATATTGAATCCAGGAAGTTC	Gene specific sequencing	59.2
	qRT-CCD4-For	CGGAGGCGGAGGAGGATGATG	Gene specific for q-PCR	62.4
	qRT-CCD4-Rev	CGGCGGCGACGATTTCAAG	Gene specific for q-PCR	61.0
<i>CCD8</i>	CCD8 For	CACCATGGCTTCTTTGATCACAACCAAAGC	Gene specific	67.4
	CCD8 Rev	TTAATCTTTGGGGATCCAGCAACCATGC	Gene specific	66.1
	CCD8-Int-For	CCTTGAATCCGACGCTTACAAAGC	Gene specific sequencing	60.0
	CCD8-Int-Rev	CGTGACCATGGGAAGAACGTAGGGT	Gene specific sequencing	62.9
	qRT-CCD8-For	TGAACCATTCCTTCGTGCCTCG	Gene specific for q-PCR	57.4
	qRT-CCD8-Rev	GCAACCATGCAAGCCATAAGGA	Gene specific for q-PCR	58.6
<i>NCED2</i>	NCED2 For	CACCATGGTTTCTCTTCTTACAATGCCG	Gene specific	63.4
	NCED2 Rev	TTATAATTGATCAACGAGTTCATTCTGAATCCA	Gene specific	62.5
	NCED2_internal primer_1	GAGATTGGTTCAGGAAAAACGATTG	Gene specific for sequencing	57.0
	NCED2_int_reverse	GTAAACCGGCATGCGTAGCTAG	Gene specific for sequencing	54.5
<i>NCED3</i>	NCED3 For	CACCATGGCTTCTTTACGGCAACG	Gene specific	56.0

	NCED3 Rev	TCACACGACCTGCTTCGCCAAATC	Gene specific	63.9
	NCED3-Int-For	TCAAAGGAGTGTATGTGCGCAACG	Gene specific for sequencing	60.8
	NCED3-Int-Rev	CGTTGAAAATTGAGTCTGGTGGAGT	Gene specific for sequencing	57.5
	qRT-NC3-For	TCCTCTGTTTCGTTACGACG	Gene specific for q-PCR	55.1
	qRT-NC3-Rev	CGTACGGAACCCCTTGACGGA	Gene specific for q-PCR	57.1
NCED5	NCED 5 For	CACCATGGCTTGTTCTTACATATTAACACC	Gene specific	60.1
	NCED 5 Rev	TTAAGCCTGGTTTAACATATCCGCCG	Gene specific	62.0
	NCED5_internal primer_1	GCTATTTTACGCACGTGGTTTATTCTG	Gene specific for sequencing	59.1
	NCED5_int_reverse	CCGAGTTGTTTCTCTTGAACCAATCTC	Gene specific for sequencing	59.5
NCED6	NCED 6 For	CACCATGCAACACTCTCTTCGTTCTG	Gene specific	60.8
	NCED 6 Rev	TCAGAAAACCTTGTTCCCTCAACTGATTCT	Gene specific	58.2
	NCED6_internal primer_1	GCTCGAGCTGGGATCGGTCTAG	Gene specific for sequencing	59.0
	NCED6_int_reverse	GTGAAGCTCGCCGATTGGTTTAG	Gene specific for sequencing	58.9
	35s-F3	CAATCCCCTATCCTTCGCAAGACCC	Specific for 35S promoter in pMDC-32	63.5
M13	M13 Fwd	GTAAAACGACGGCCAG	Specific for the M13 region in pENTRD	43.6
	M13 Rev	CAGGAAACAGCTATGAC	Specific for the M13 region in	36.2

			pENTRD	
<i>UBQ</i>	UBQ10-Fq	GCTCCGACACCATCGACAACG	Reference gene in q-PCR	59.9
	UBQ10 Rq	CTGAGGACCAAGTGGAGGGTGA	Reference gene in q-PCR	61.0
<i>PSY</i>	PSY For	TGCGGTGAAGTTTGCGCTGA	Gene specific for q-PCR	59.9
	PSY Rev	TGAAGCATTTGGCCCATCCA	Gene specific for q-PCR	58.4
<i>bLYC</i>	bLYC For	TGGTAGCGCTGCTCTTTTGGA	Gene specific for q-PCR	57.4
	bLYC Rev	ACCAGCAGGACCACCACCA	Gene specific for q-PCR	55.8
<i>PDS</i>	PDS For	GTCGGTCACGCGCTCAGGTA	Gene specific for q-PCR	58.4
	PDS Rev	CGAGATGCTGACATGGCCAGA	Gene specific for q-PCR	58.3
<i>ZDS</i>	ZDS For	CCATCGTCACGAGGCCTAGAA	Gene specific for q-PCR	56.5
	ZDS Rev	TGTGTATGAACCGGCGAGGA	Gene specific for q-PCR	56.2
<i>BCH1</i>	BCH1 For	GGCACGCTTCTCTATGGAATATGCATGA	Gene specific for q-PCR	63.9
	BCH1 Rev	GAATCCATAAGAGAGGAGACCAATCGCT	Gene specific for q-PCR	61.2
<i>LUT1</i>	LUT1 For	CGAAATCCCAATCATGGGTCA	Gene specific for q-PCR	56.8
	LUT1 Rev	GCACCTCCGAGGAGATCAGC	Gene specific for q-PCR	56.0
<i>ZEP</i>	ZEP For	ATGACCGGCTTCGAGAGTGG	Gene specific for q-PCR	56.4
	ZEP Rev	TTCCGACGATGCAAGGTTGA	Gene specific for q-PCR	56.3
<i>VDE</i>	VDE For	ACCGCTCCGCTGTTGCTAAA	Gene specific for q-PCR	57.3
	VDE Rev	TGGCAATGCACTTTGCGAGT	Gene specific	56.5

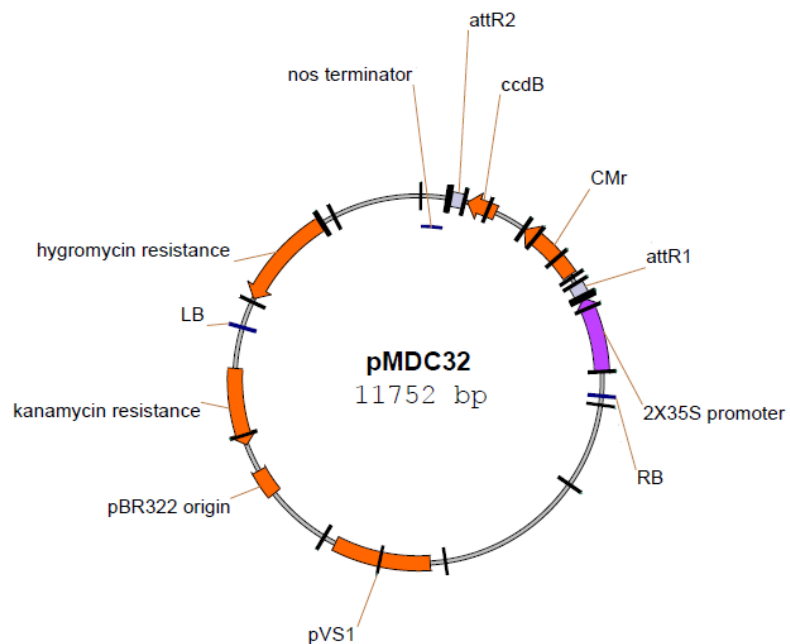
			for q-PCR	
<i>ABA2</i>	ABA2 For	ACGGTTGATGATGTAGCGAACGCTGTT	Gene specific for q-PCR	64.4
	ABA2 Rev	CATCTGAAGACTTTAAAGGAGTGGTTAG	Gene specific for q-PCR	54.7

### 2.3 Cloning of *CCD* and *NCED* into expression vectors

To clone *CCD* and *NCED* genes into the expression binary pMDC-32 vector, the amplified full-length cDNA of each gene was purified using the QIAquick PCR purification kit (QIAGEN, Canada), and was then inserted into the Gateway pENTRD vector (the entry vector; Life Technologies) using 0.5  $\mu$ L of BP clonase enzyme, 5 ng/ $\mu$ L of the PCR product, and 0.5  $\mu$ L of the pENTRD vector; this was transformed into chemically competent One Shot® TOP10 *E.coli* cells using the freeze thaw technique described by (Wise et al., 2006). Positive clones were identified using PCR, and those showing the expected amplicon size, were confirmed by sequencing. The cDNA was then transferred from pENTRD to pMDC-32 (Curtis and Grossniklaus, 2003) by recombination using 0.5  $\mu$ L LR clonase, according to manufacturer's instructions. The pMDC-32 vector contains the CaMV 35S constitutive promoter, NOS terminator, kanamycin as the bacterial selectable marker, and hygromycin as the plant antibiotic resistance marker (Figure 2). This vector was selected for its suitability with Gateway® cloning. Recombination of the gene from pENTRD to pMDC-32 was performed using Gateway® LR Clonase® II enzyme mix according to the manufacturer's instruction (Life Technologies, USA). Transformation of the TOP10 *E.coli* cells was carried out using electroporation. In order to eliminate background false positive colonies (due to the presence of the same antibiotic resistance marker gene in the entry vector and destination vector), the pENTRD plasmid containing the cDNA was subjected to digestion using single-cutting restriction enzymes (New England Biolabs, USA) to linearize the plasmid. The enzymes used for each construct are listed in Table 3. Once the cDNA was recombined into pMDC-32 the positive TOP10 cells were screened by PCR and those



showing the expected band size were confirmed further by sequencing. The overexpression constructs were then transformed into *Agrobacterium tumefaciens* cells (GV3101 strain; containing rifampicin and gentamycin resistance) using electroporation. Electroporation was performed using the Gene Pulser® Cuvette (BioRad Laboratories, Canada) with 0.1 cm electrode gap using MicroPulser™ (BioRad Laboratories, Canada). The electroporation setting used for TOP10 and GV3101 was 1.79 kV and 2.18 kV, respectively, for six milliseconds.



**Figure 2: pMDC32 the binary vector in the Gateway® cloning.** pMDC-32 was used as the destination vector in the cloning of the *CCD* and *NCED* genes due to the presence of the dual CaMV 35S promoter region in the vector and compatibility with the Gateway cloning system.

**Table 3: A list of restriction enzymes used for vector linearization.** Each construct was digested using a specific enzyme listed below to linearize the pENTR-D vector in the LR cloning.

Genes	Restriction enzymes for each construct
<i>CCD4</i>	ApaI
<i>CCD8</i>	ApaI
<i>NCED2</i>	KpnI
<i>NCED3</i>	ApaI
<i>NCED5</i>	ApaI
<i>NCED6</i>	EcoRI

### 2.3.1 Plasmid extraction

Plasmid extraction from the *E. coli* and *A. tumefaciens* colonies was performed using the alkaline lysis plasmid miniprep protocol. The colonies selected for plasmid extraction were inoculated in 3 mL Luria Bertani (LB) broth containing the appropriate antibiotics. The culture broth was grown overnight at 37 °C and 28 °C for *E. coli* and *A. tumefaciens*, respectively in an incubator shaker. The overnight culture was then transferred to 1.5 mL Eppendorf tubes and spun down at 17,500 g in a microcentrifuge for 1 min. The supernatant was decanted and the pellet was resuspended in 200 µL of ice-cold solution I (containing RNase), and vortexed well. Next, 200 µL of solution II containing SDS was then added to the tube and mixed by gently inverting. Subsequently, 200 µL of ice-cold solution III containing glacial acetic acid was added to the tube and mixed by gently inverting and then chilled on ice for 5-10 min. The tubes were then centrifuged at 17,500 g for 5 min and the supernatant containing the DNA was transferred to a fresh tube. Approximately two volumes of ice cold 100% ethanol were added to the tubes and the DNA was allowed to precipitate at -20 °C for 10 min. This mixture was then centrifuged at 17,500 g for 10 min and the ethanol was decanted. The plasmid DNA pellet was washed with 200 µL of 70% (v/v) ethanol and the pellet was air-dried completely before being resuspended in 50 µL of fresh MilliQ water (refer to Table 4 for solution compositions).

**Table 4: Composition of solutions used in plasmid extraction.**

<b>Solutions</b>	<b>Compositions</b>
Solution I (1 L)	Glucose (9 g), TRIS (3.02 g), EDTA (3.72 g), RNase (0.1 mg/mL), pH 8.0
Solution II (10 mL), prepared fresh	2N NaOH (1 mL), 10% SDS (1 mL), MQ (8 mL)
Solution III (1 L)	CH <sub>3</sub> COOK (294.45 g), Glacial acetic acid (115 mL), pH 5–6.

### **2.3.2 Arabidopsis transformation**

Four-week-old healthy Arabidopsis plants were used for *A. tumefaciens*-mediated transformation. Plants showing inflorescences were selected for transformation by the floral dipping procedure as described by Clough and Bent (1998). A second floral dip was performed at a one-week interval after the first floral dipping was performed (Clough and Bent, 1998). Following transformation, the plants were allowed to mature to senescence and seeds were then collected for screening.

### **2.3.3 Screening for Arabidopsis transformants**

Seeds were collected from Arabidopsis plants previously “transformed” as above, and surface sterilized using a solution of 70% (v/v) ethanol and 5% (v/v) Triton X-100 (Sigma Aldrich, USA), for 10 minutes with gentle shaking. The seeds were washed three times with 95% ethanol and transferred to a sterilized filter paper, where they were allowed to dry for about 30 min. The seeds were screened for positive transformants on 0.5 × Murashige and Skoog Basal salt mixture (MS) medium (*PhytoTechnology* Laboratories, USA) containing 1.5% (w/v) sucrose, 0.8% (w/v) phytoagar (Sigma-Aldrich, Germany) and adjusted to pH 5.8 using 1M KOH. The medium contained 25 µg/mL hygromycin (Life Technologies, USA) as the selectable marker. Two weeks after the seeds were sown in MS medium, positive seedlings were selected based on their survival post germination. The transgenic seedlings were transplanted to soil and the presence of the transgene was confirmed using a rapid PCR protocol (Edwards et al., 1991) involving punching leaf samples from the rosette leaves, extracting DNA using extraction buffer (Refer to Table 5 for composition) and performing PCR amplification of the transgene using one vector-specific primer and one transgene-specific primer.

**Table 5: Composition of buffers used in the DNA extraction from leaves**

<b>Extraction buffer</b>	<b>100 mL</b>
Millipore water	65 mL
200 mM Tris pH 8.0	20 mL of 1M Tris
250 mM NaCl	5 mL of 5 M NaCl
25 mM EDTA	5 mL of 0.5 M EDTA
0.5% SDS	5 mL of 10 % SDS

## 2.4 Quantitative real time PCR

T1 seeds were collected from transformed plants and sown on MS medium containing antibiotics (hygromycin; 25 µg/mL) for further screening using antibiotic selection. The T1 plants that were resistant to hygromycin were transferred to soil and screened for the presence of the transgene using PCR. The seeds from each independent transgenic T1 line were collected (T2 seeds). The T2 seeds were sown onto MS medium containing 25 µg/mL of hygromycin and post germination, the seedlings that successfully grew on this medium were selected. These seedlings were then transplanted to soil, further screened by PCR, and subsequently seeds (T3 seeds) were collected from these T2 lines. The T3 seeds were sown on MS medium containing the antibiotic. On the basis of segregation, the T3 seeds did not show any segregation thus indicating that the lines were homozygous. Three-week-old plants were used for RNA extraction as described previously (Section 2.2). The quality of RNA was verified by spectrophotometry ( $A_{260/280}$  ratio of 1.8 – 2.0) and the samples were analyzed on a 1% (w/v) agarose gel in 1× TAE buffer. DNase treatment was performed using the TURBO DNase kit, as mentioned in section 2.2. cDNA synthesis was carried out using the qScript cDNA Supermix that contains a blend of oligo dT and random primers (Quanta Biosciences, USA).

Approximately 1 µg of total RNA was used per cDNA reaction. After cDNA synthesis, the cDNA samples were diluted four times in millipore water. The primers were used at a final concentration of 5 µM each for every 10 µL reaction. A list of the primers used for qRT-PCR is listed in Table 2. PerfeCta® SYBR® Green FastMix® (Quanta Biosciences, USA) was added to a final concentration of 1×. The fast 2-step cycling program recommended by the kit (95 °C for 30 s for one cycle, and 95 °C for 5 s and 60 °C for 30



s for 45 cycles) was used for the quantitative amplification of the cDNA samples (Bio-Rad, CFX96™). Melt-curve analysis was performed (65-95 °C for 5 s, with a 0.5 °C increment) for each primer. For molecular characterization of genes in the carotenoid biosynthesis pathway, the cycling parameters were modified based on the  $T_m$  of the primers used for qPCR (Table 2).

#### **2.4.1 Normalization of relative amounts of gene expression using reference genes**

The qPCR expression data were normalized using a reference gene. For this, three candidate reference genes, namely Polyubiquitin (UBQ10; AT4G05320), Protein phosphatase 2A subunit A3 (PP2AA3; AT1G13320), and Actin (Act2; AT3G18780) were tested for stability of gene expression. The gene that had most stable gene expression was UBQ10, unlike the other two genes that showed variability in expression; hence UBQ10 was selected as the most suitable reference gene. The  $2^{(-\Delta\Delta Ct)}$  method was used to normalize genes, as described by the instrument manufacturer (Bio-Rad, CFX96™).

### **2.5 Extraction of carotenoids from Arabidopsis leaves and HPLC analysis**

The levels of carotenoids in the leaves of Arabidopsis CCD4 and CCD8 transformants were assessed to observe any changes as a result of the overexpression of the CCD genes in the plants. Four-week-old rosette leaves were used for the carotenoid extraction as described by (Yu et al., 2012). Four biological replicates were used for every line. Frozen tissue samples were ground using a mortar and pestle and with the addition of liquid nitrogen. The ground leaf samples were collected in pre-weighed

plastic 15 mL conical tubes. The tubes were reweighed after the samples were added to determine the sample weight (range from 50-100 mg). The protocol was carried out under low light conditions due to the sensitivity of carotenoids to light. Next, 3 mL of a hexane/acetone/ethanol (50:25:25) solution was added to the tubes, which were vortexed for 5 min at 1200 g in a VWR multi tube vortexer (VWR; USA). The tubes were centrifuged for 10 min at 1800 g at 4 °C. The supernatant was then transferred to conical glass centrifuge tubes (Kimble Chase; USA). Re-extraction of the pellet was performed by adding 3 mL of the hexane/acetone/ethanol solvent and vortexing and centrifuging the samples as mentioned earlier. The samples were further evaporated under nitrogen stream. Saponification of fats in the sample was carried out by adding 5 mL of methanolic KOH (80% MeOH (v/v), 10% (w/v) KOH). Next, the samples were vortexed and placed in a hot water bath that was set to 80 °C for 15 min. The samples were then cooled on ice and 2 mL of petroleum ether was added to the samples, which were then vortexed and centrifuged for 15 min at 450 g at 4 °C. The ether layer (top layer) containing carotenoids was transferred to a fresh glass tube. Re-extraction was repeated by adding another 3 mL of ether and repeating the aforementioned steps. Once the ether layers were pooled, evaporation under nitrogen stream was performed. Once the sample was dry, 200 µL of resuspension solvent containing acetonitrile/dimethylchloride/methanol (50:40:10) with 0.5% (w/v) BHT (butylated hydroxytoluene) was added. The samples were filtered through a 0.2 µm pore size 13 mm syringe filter with mini spike outlet (PALL Life Sciences Acrodisc® Part # 4550T; USA) into amber HPLC vials containing inserts (Agilent; Germany). The samples were subjected to analysis by HPLC (Agilent Technologies 1200 series) employing a YMC

“Carotenoid Column” – a reverse phase C<sub>30</sub>, 5 µm column (4.6 × 250 mm; Waters Ltd, Mississauga, Canada) with a column temperature of 35 °C. Mobile phases consisted of methanol (A) and tert-methyl butyl ether (B). The gradient elution used with this column started at 95% A and 5% B, and then followed by a linear gradient to 35% A and 65% B in 25 min. A flow rate of 1.2 mL/min was used, and the eluate was monitored at 450 nm. Analysis of carotenoid peaks was performed using the Agilent Chemstation software. Peaks were identified by their retention times and absorption spectra compared to those of known standards (lutein, β-carotene, and violaxanthin). Quantification of carotenoids was conducted using standard curves of the authentic standards in order to determine the retention time (RT) of the compounds.

## **2.6 Analysis of volatile emissions using Gas Chromatography-Mass Spectroscopy**

The volatile profiles of the transgenic plants were compared to that of WT plants by collecting the volatiles from the plants over a 24 h period. A set of eight potted plants at the flowering stage (six-week-old) per line were enclosed in a cylindrical 46 cm × 26 cm glass chamber (Figure 3). The chamber was placed in a growth chamber that was maintained at 22 ± 3 °C with a photoperiod of 16 h and a relative humidity of 55%. Before the plants were placed in the chamber, a Petri dish filled with activated carbon (Fisher Scientific; Canada) was placed in the chambers for about 24 h to absorb any traces of ambient volatiles. The volatiles were collected over a 24 h period using compressed air at a flow rate of 100 mL/min. A Porapak Q 75/150 polydivinylbenzene column (Cat. # 226-115; SKC Inc., USA) was connected at the outlet of the chamber to collect the volatiles. Every collection was performed along with a WT control. After a 24

h period, the samples were eluted from the columns using 3 mL HPLC grade DCM (dichloromethane). The eluent was then concentrated to exactly 0.25 mL by passing the samples under a stream of nitrogen gas. 2-Octanone was added to the samples as the internal standard at a final concentration of 20 µg/mL. The samples were analyzed using an Agilent Technologies Inc. fused silica capillary column (DB-5MS + DG; 5% (w/v) phenylmethyl silicone; 30 m length + 10 m Duraguard × 0.25 mm i.d.; film thickness 0.25 µm) and an Agilent Technologies 7890A chromatograph equipped with an Agilent Technologies 5975C inert XL EI/CI MSD Triple-Axis Detector. The carrier gas used was Helium (12.445 psi; 1.2315 mL/min). The voltage used in the EMV mode was relative and the resulting EMV was 1376. The oven temperature was maintained at 30 °C for 1 min, then increased at 5 °C/min to 200 °C, and then held for 1 min at this temperature. The total run time for each sample was 36 min. Two microliters of plant volatile samples were injected using an autosampler into the gas chromatograph (GC) in the pulsed splitless mode (25 psi until 0.5 min; the purge flow to the split vent was adjusted at 40 mL/min for 1 min). Volatile compounds in Arabidopsis leaves were identified by comparison of the mass spectra obtained from authentic standards and additionally confirmed with mass spectroscopy (MS) data with the NIST08 and W8N08 libraries (John Wiley and Sons, Inc., New York, NY). Analysis of the volatile profiles was performed using the AMDIS\_32 software (version 2.68; Jan 28 2010; Build, 126.47). Compounds corresponding to each peak were identified using the NIST Mass Spectral database software (version 2.0 f; Build Apr 1 2009).



**Figure 3: Collection of the headspace volatiles from six-week-old Arabidopsis plants.** Headspace volatiles were collected over a 24 h period from flowering Arabidopsis plants onto Porapak Q columns.

### 2.6.1 Analysis of volatile compounds by comparison with standards

In addition to identification of the volatile compounds using the libraries, the profiles were compared with standards in order to confirm the identity of the compounds. The following standards were run: 1)  $\beta$ -ionone (SAFC; Lot # MKBH4976V); 2) Oxoisopherone (SAFC); 3)  $\beta$ -ionol (Sigma Aldrich Lot# E3844381V); 4)  $\alpha$ -ionone (SAFC, Lot# MKBD1295V); 5)  $\beta$ -damascenone (Sigma Lifesciences, Lot# BCBC9470V); 6) Theaspirane (SAFC, Lot# 56696AJ); 7) Isophorone (SAFC); 8) Caryophyllene (Sigma Lifesciences, Lot# BCBG0948V); 9) Limonene (Sigma Aldrich, Lot# BCBF5924V); 10) Dihydro- $\beta$ -ionone (SAFC); 11)  $\beta$ -cyclocitral (SAFC, Lot# MKBF1272V).

### 2.7 Assessing insect feeding damage

Overwintering adult flea beetles (*P. cruciferae*) that emerged during the early spring were collected in net sweeps from pesticide free canola fields at the Southern Crop Protection and Food Research Centre (SCPFRC, London, Ontario). All experiment trials were carried out using the same generation of flea beetles (overwintered adults). Once the insects were collected for the experiments, they were maintained indoors in cages on a diet of radish leaves and water for about 1-2 days. The cage was placed in an evenly lit growth room under constant temperature ( $23\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ ), a relative humidity of  $65\% \pm 3\%$  and a photoperiod of 16 h (Wei et al., 2011). The insect feeding bioassay experiments were performed in the same growth chambers that were used for the volatile collections ( $22 \pm 3\text{ }^{\circ}\text{C}$  with a photoperiod of 16 h), and the plants used were six-week-old. Four potted plants per line were placed in the glass chamber. The openings of the chambers were closed with a thin mesh material. One of the outlets of the chamber was connected

to a vacuum pump and the other outlet was connected to a tube, which was attached to a nozzle. The vacuum created in the chamber facilitated suction of the beetles into the chamber. Each chamber containing four potted plants was enclosed with 200 flea beetles for a 24 h period. After the 24 h period, the rosette leaves of the damaged plants were clipped and taped onto paper, after which, the sheets of papers with leaves were scanned (EPSON, USA). Leaf damage was analyzed using the Corel Photo Paint 12 software (CorelDraw Graphics Suite 12). The total leaf area consumed was calculated based on the total number of pixels that represented the damaged area. An average of the percentage of leaf damage was taken for the four plants. At least three trials were performed for every line, which was always run with WT control plants.

## **2.8 Statistical analysis**

All statistical analyses were performed using the Microsoft Excel T-test calculation to determine the significant differences between the control and test groups. *P* values of 0.01 (99% confidence interval) and 0.05 (95% confidence interval) were used to measure the statistical differences.

## Chapter 3: Results

A previous study in Dr. Hannoufa's laboratory suggested that constitutive overexpression of *CCD1*, one of the genes in the nine-member *CCD* gene family, in *Arabidopsis* resulted in changes in the volatile apocarotenoid profiles of these plants (Wei et al., 2011).  $\beta$ -Ionone was found to be present in high levels in the *CCD1* overexpression plants as compared to WT. Further, when the *CCD1* plants were subjected to insect feeding by the crucifer flea beetle, they showed significantly lower feeding damage compared to the WT plants. This study prompted me to explore the effects of overexpressing the remaining eight *CCD* genes in the family on flea beetle feeding on *Arabidopsis*.

### 3.1 Generating transgenic *Arabidopsis* plants overexpressing *CCD* and *NCED* genes

Overexpression constructs were prepared for six genes, namely, *CCD4*, *CCD8*, *NCED2*, *NCED3*, *NCED5*, and *NCED6*. Of these, three (*CCD4*, *CCD8*, and *NCED3*) were transformed successfully into *Arabidopsis*. Two of the other constructs (*NCED2* and *NCED6*) were transformed into *Arabidopsis*, but T<sub>0</sub> seeds were not screened for transformants, and *NCED5* was not transformed to *Arabidopsis*.

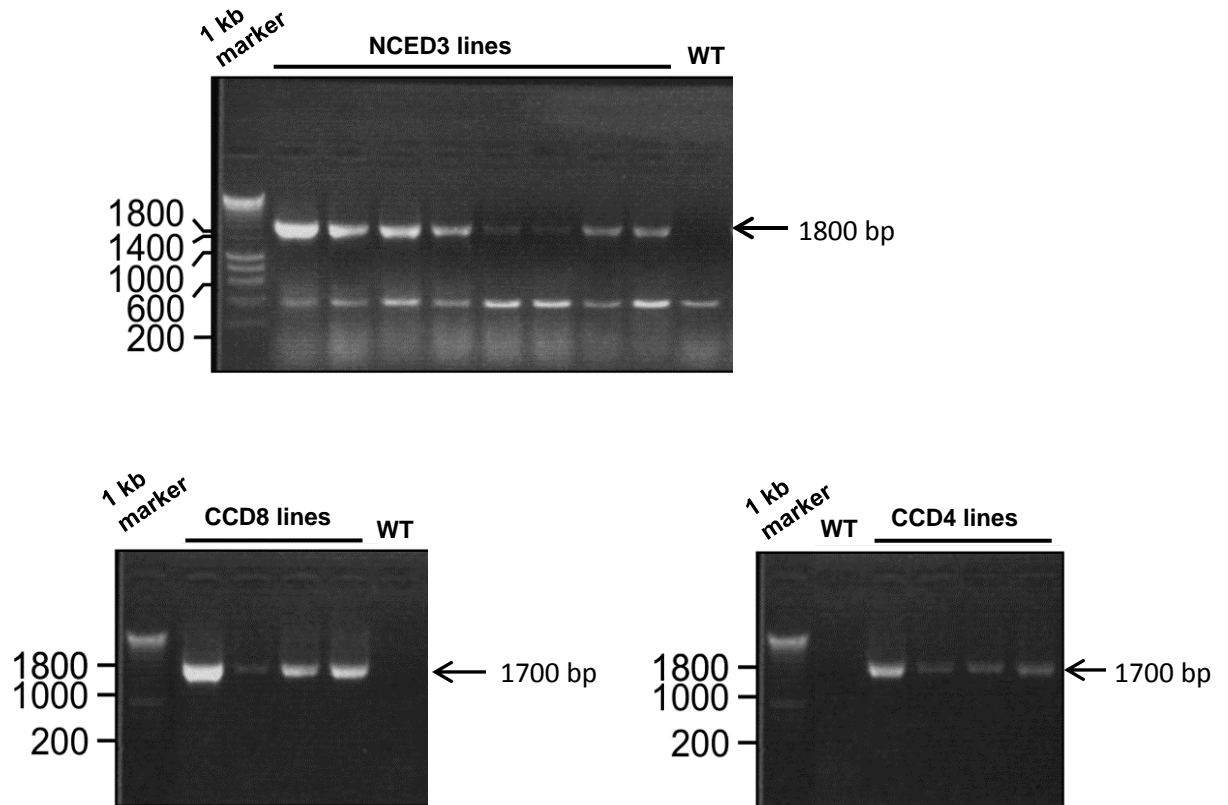
The T1 plants harboring the *CCD4*, *CCD8*, and *NCED3* overexpression constructs were screened using the antibiotic resistance marker (hygromycin). The positive seedlings that could grow on the antibiotic MS media were then transplanted to soil (Figure 4). The T1 plants did not show any visible developmental differences compared



to the WT. PCR was performed to confirm the presence of the transgene (Figure 5), and positive transformants showing the presence of the transgene were transplanted to soil.



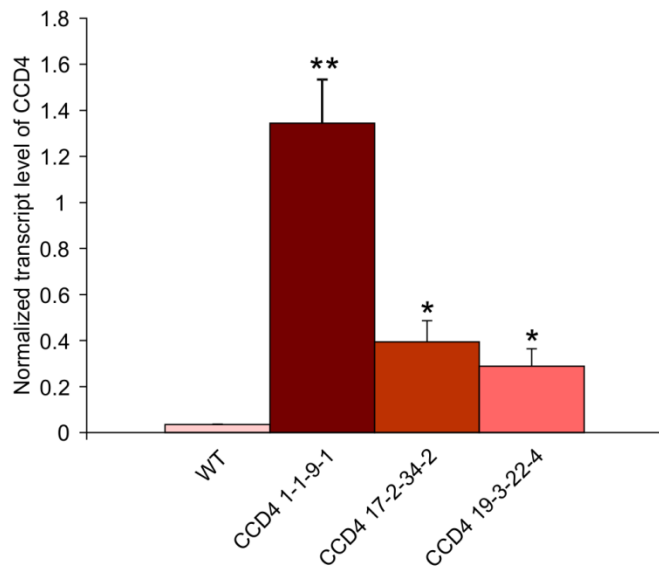
**Figure 4: Four-week old transgenic T1 plants.** The transgenic seedlings that were positive when grown on MS media containing hygromycin were transplanted to soil when they were two-week-old. These plants did not show any obvious phenotypic differences compared to the WT plants.



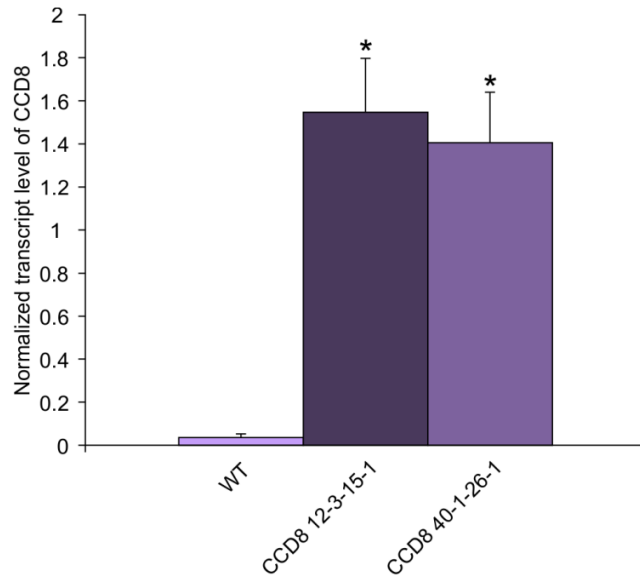
**Figure 5: Screening for transgenic Arabidopsis plants overexpressing CCD4, CCD8, and NCED3 genes by PCR.** Genomic DNA from the positive transgenic seedlings that successfully germinated and grew on medium supplemented with hygromycin (25  $\mu\text{g/mL}$ ) was confirmed by PCR using the 35S-F3 forward primer and gene-specific reverse primers.

### **3.2 Expression analysis of CCD4 and CCD8 transgenic Arabidopsis plants**

In order to confirm the transcript levels of the *CCD4* and *CCD8* transgenes, qRT-PCR was performed on the T3 generation of the transgenic plants. The transcript levels of six independent lines for each transgene were tested and the transcript levels were compared to that of WT plants. In the case of *CCD4*, of the six lines that were screened, three lines had significantly higher *CCD4* transcript levels compared to WT (Figure 6). With regard to *CCD8*, of the six lines tested, two transgenic lines were shown to have significantly higher *CCD8* transcript levels compared to the WT (Figure 7).



**Figure 6: Transcript level of CCD4 and CCD8 in three Arabidopsis transgenic lines and WT.** Each bar represents the mean of four biological replicates. The error bars represent the standard error of mean. The means of the WT and each transgenic line were compared using the two-tailed T-test. The level of significance indicated by “\*” represents  $P \leq 0.05$  and “\*\*” represents  $P \leq 0.01$ .



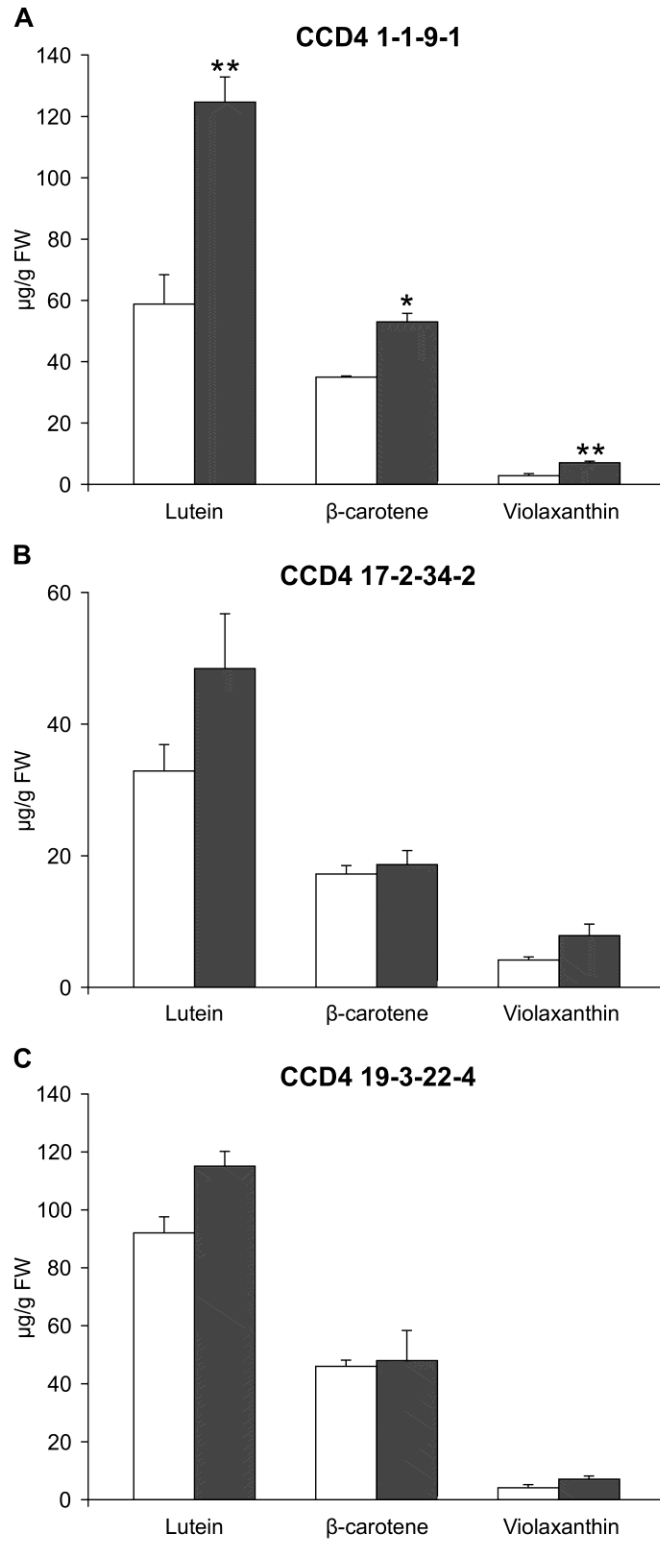
**Figure 7: Transcript level of CCD8 in two Arabidopsis transgenic lines and WT.**

Each bar represents the mean of four biological replicates. The error bars represent the standard error of mean. The means of the WT and each transgenic line were compared using the two-tailed T-test. The level of significance indicated by “\*” represents  $P \leq 0.05$ .

### **3.3 Effects of overexpression of *CCD4* and *CCD8* on the carotenoid content of *Arabidopsis* leaves**

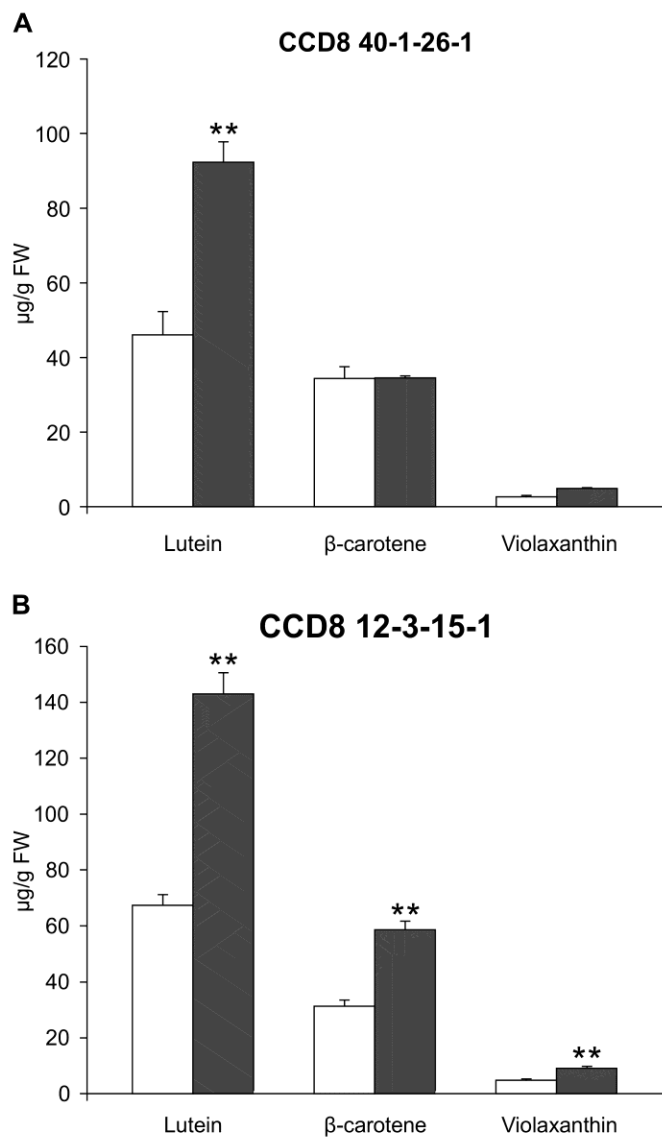
Since the CCD family of enzymes play an important role in regulating the steady-state level of carotenoids in plants, I wanted to assess the effects of *CCD* overexpression on the carotenoid content of the leaves. I anticipated that overexpression of *CCD* genes would result in lower levels of carotenoids in the leaves. Four-week-old rosette leaves of the *CCD4* and *CCD8* overexpression lines were used to analyze the carotenoid content of the predominant carotenoids in leaves – lutein,  $\beta$ -carotene, and violaxanthin using HPLC.

Surprisingly, HPLC analysis of carotenoids showed enhanced levels of three carotenoids in the leaves of transgenic plants compared to WT. This accumulation of carotenoids in the leaves was observed in the case of both the *CCD4* and *CCD8* transgenic lines. The *CCD4* line with the highest *CCD4* transcript levels, CCD4 1-1-9-1, showed a significantly higher level of lutein,  $\beta$ -carotene, and violaxanthin compared to WT (Figure 8 A). This trend was observed in the other two *CCD4* transgenic lines 17-2-34-2 and 19-3-22-4 lines; however, the difference was not statistically significant in the case of these lines (Figure 8 B & C). In the case of *CCD8*, CCD8 40-1-26-1 only showed a significant accumulation of lutein but not the other two carotenoids (Figure 9 A), whereas CCD8 12-3-15-1 showed significantly higher levels of all the three carotenoids compared to WT (Figure 9 B). The levels of accumulation appeared to be proportional to the transcript level of *CCD4* and *CCD8* genes.



**Figure 8: Analysis of total carotenoids in leaves of three lines (A, B, and C) of transgenic CCD4 Arabidopsis plants.** The white bars represent the WT control and the grey bars represent the overexpression lines. Each bar represents the mean of at least three biological replicates. The error bars represent the standard error of mean. The means of the WT and each transgenic line were compared using the two-tailed T-test. The level of significance indicated by “\*” represents  $P \leq 0.05$  and “\*\*\*” represents  $P \leq 0.01$ .





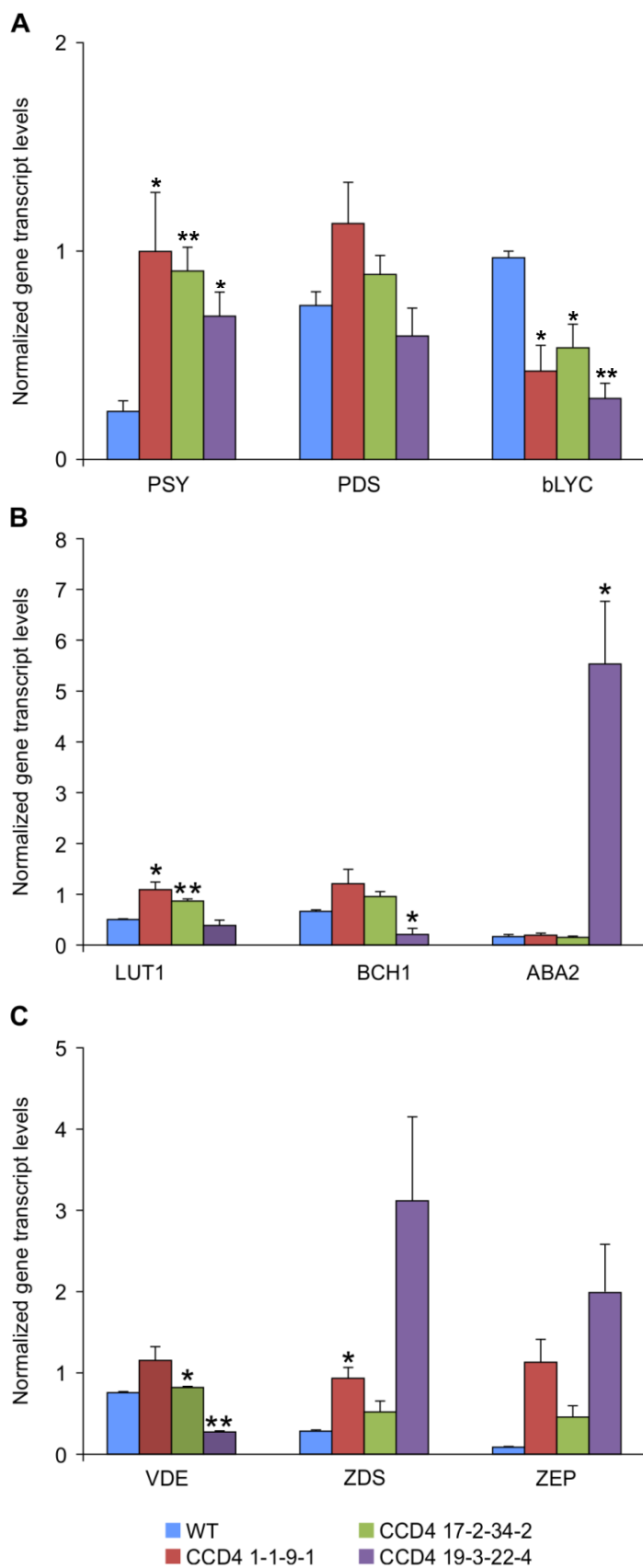
**Figure 9: Analysis of total carotenoids in the leaves of two lines (A & B) of transgenic CCD8 Arabidopsis plants.** The white bars represent the WT control and the grey bars represent the overexpression lines. Each bar represents the mean of at least three biological replicates. The error bars represent the standard error of mean. The means of the WT and each transgenic line were compared using two-tailed T-test. The level of significance indicated by “\*\*” represents  $P \leq 0.01$ .

### 3.4 Analysis of transgenic *CCD4* and *CCD8* plants for expression of carotenoid genes

#### 3.4.1 Expression analysis of carotenoid genes in transgenic *CCD4* lines

On the basis of the results of the carotenoid quantification in leaves, it was necessary to investigate expression of genes involved in the carotenoid biosynthesis pathway. I was interested to determine if the levels of gene expression were proportional to the levels of accumulation of the carotenoids in the leaves of the transgenic plants compared to the WT plants. To take a closer look at the changes in the gene expression levels in the transgenic lines, q-PCR was performed on the key regulatory genes in the carotenoid biosynthesis pathway. The genes analyzed were those involved in the pathway prior to the lycopene branching step and those involved in the synthesis of the carotenoids at the  $\beta,\beta$  and  $\beta, \epsilon$  branches (Figure 1). The genes that were analyzed in the pathway prior to lycopene were *PSY* and *PDS*. The genes involved in the pathway at the  $\beta, \beta$  and  $\beta, \epsilon$  branches are *LUT1*, *BCH1*, *VDE*, *ZEP*, *ZDS*, *bLYC*, and *ABA2* (Figure 1). qPCR analysis revealed that the transcript levels of *PSY* in all three *CCD4* overexpression lines was significantly greater than in WT. In the case of the *PDS* gene, *CCD4* overexpression lines exhibited a trend towards higher *PDS* transcript levels compared to WT, although the difference was not statistically significant. It was interesting to note that the transcript level of *bLYC* in the *CCD4* transgenic lines was significantly lower than in WT (Figure 10 A). In the case of *LUT1*, the transcript level was significantly greater in *CCD4* 1-1-9-1 and *CCD4* 17-2-34-2 lines than in WT; however, *CCD4* 19-3-22-4 did not show any statistically significant differences compared to the WT. The transcript levels of *BCH1* in *CCD4* 1-1-9-1 and 17-2-34-2 did not show any differences

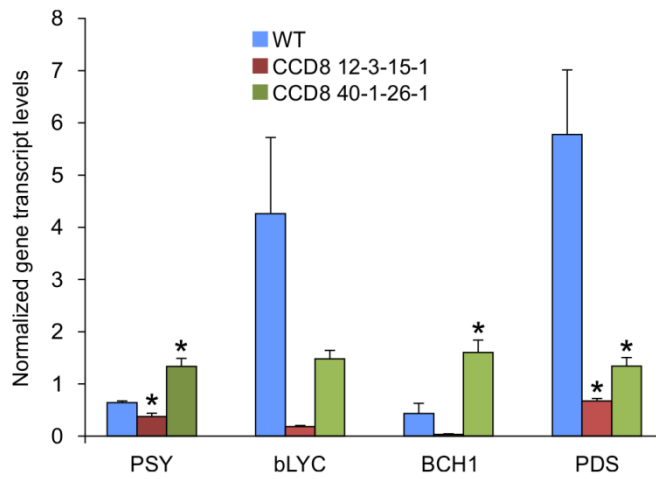
compared to WT; however, the transcript levels were significantly lower in CCD4 19-3-22-4, compared to WT. It was interesting to observe that the *ABA2* transcript levels showed a significant increase in CCD4 19-3-22-4 compared to the WT; there were no noticeable differences in *ABA2* transcript levels in the other two CCD4 transgenic lines compared to the WT (Figure 10 B). Although *VDE* did not show any differences in CCD4 1-1-9-1 compared to the WT, *VDE* transcript levels were significantly increased in CCD4 17-2-34-2 and decreased in CCD4 19-2-34-2. *ZDS* showed a significant increase in CCD4 1-1-9-1, but no differences were observed in the other two CCD4 transgenic lines compared to the WT. There was no specific pattern observed with respect to the transcript levels of *ZEP* in the transgenic lines (Figure 10 C).



**Figure 10: qRT-PCR analysis of genes involved in the carotenoid biosynthesis pathway in CCD4 Arabidopsis plants.** qRT-PCR was performed on the genes involved in the carotenoid pathway in the CCD4 lines – CCD4 1-1-9-1, 17-2-34-2, and 19-3-22-4 as well as WT. The data was normalized to the UBQ10 gene. Each bar represents the mean of at least three biological replicates. The error bars represent the standard error of mean. The means of the WT and each transgenic line were compared using two-tailed T-test. The level of significance indicated by “\*” represents  $P \leq 0.05$  and “\*\*” represents  $P \leq 0.01$ .

### 3.4.2 Expression analysis of carotenoid genes in transgenic CCD8 lines

The patterns of up-regulation of the carotenoid biosynthesis genes in the CCD8 lines varied from that observed in the *CCD4* lines. The carotenoid genes that were tested in the CCD8 lines were *PSY*, *PDS*, *bLYC*, and *BCH1*. In CCD8 12-3-15-1, the transcript levels of *PSY* was significantly lower than that of WT, whereas in the case of CCD8 40-1-26-1, the levels were significantly higher than that in WT (Figure 11). The transcript levels of *PDS* were significantly lower in both the CCD8 lines compared to WT, thus suggesting that *CCD8* overexpression may have an effect on the expression of *PDS*. Although the pattern of transcript levels of *bLYC* was lower in the two transgenic CCD8 lines than WT, this difference was not statistically significant. The transcript level of *BCH1* was significantly higher in CCD8 40-1-26-1 compared to WT; however, there were no differences in the levels of *BCH1* in CCD8 12-3-15-1 and WT (Figure 11).



**Figure 11: qRT-PCR analysis of genes involved in the carotenoid biosynthesis pathway in CCD8 Arabidopsis plants.** qRT-PCR was performed on the genes involved in the carotenoid pathway in the CCD8 lines – CCD8 40-1-26-1 and 12-3-15-1 and WT. The data was normalized to the UBQ10 gene. Each bar represents the mean of at least three biological replicates. The error bars represent the standard error of mean. The means of the WT and each transgenic line were compared using two-tailed T-test. The level of significance indicated by “\*” represents  $P \leq 0.05$ .

### **3.5 Analysis of volatile apocarotenoids in transgenic CCD4 and CCD8 plants**

The volatile constituents of six-week-old flowering *Arabidopsis* plants were collected and analyzed by GC-MS. A range of volatiles, which consisted of monoterpenes, sesquiterpenes, and terpenoids, were detected. Some of the most abundant sesquiterpenes included caryophyllene,  $\beta$ -chamigrene, thujopsene,  $\alpha$ -copaene,  $\beta$ -farnesene,  $\alpha$ -bulnesene,  $\alpha$ -longipinene. The GC-MS profiles (Figure 12) did not show striking qualitative differences. In other words, there were no obvious peak differences among the CCD4 and CCD8 lines compared to WT. However, there were quantitative differences in peak intensities of the different compounds. In addition to using the NIST Mass Spectral database software to identify the various compounds corresponding to each peak in the profile, the identity of the compounds were confirmed by running standards for many of the compounds.

Based on the GC-MS data, a list of compounds detected in all the profiles was compiled (Table 6). The percentage peak area values corresponding to each compound were calculated by finding the percentage of the ratio of the peak area of the compound to that of the internal standard (2-octanone; indicated in Figure 12A) in each specific profile. In total there were 23 main plant derived volatile compounds detected in all the profiles. The percentage peak area for each compound represents the mean of three biological replicates for each line and the WT. From the list of compounds, it was clear that CCD4 1-1-9-1 displayed the widest range of compounds compared to WT and the other transgenic lines. Twelve compounds were detected in considerable amounts in the CCD4 1-1-9-1 profile, and two were found in trace amounts. Among the 12 compounds



detected in substantial amounts, one compound was found to be a volatile apocarotenoid- $\beta$ -ionone. Further, another volatile apocarotenoid detected in trace amounts is  $\beta$ -damascone. It was interesting to observe that the percent peak area of acetophenone was the highest in CCD4 1-1-9-1 compared to those of the other lines, including the WT. In addition, the percent peak area of benzaldehyde was also the highest in CCD4 1-1-9-1. Apart from the volatile apocarotenoids, other sesquiterpenes, such as  $\alpha$ -cubabene,  $\alpha$ -farnesene, and  $\alpha$ -bulnesene (peak areas 10.7%, 8.7%, and 12.2 %, respectively) were also detected.

The percentage peak area of (+)- thujopsene and *p*-cymene, both volatile sesquiterpenes, was highest in CCD4 17-2-34-2 (16.1% and 12.3 %, respectively). The peak area of (-)- myrtenol was found to be 3.3%. Myrtenol was detected only in CCD4 17-2-34-2. CCD4 19-3-22-4 was the only line that showed the presence of 3-thujen-2-ol and  $\alpha$ -thujenal. However, traces of  $\alpha$ -thujenal were observed in CCD4 17-2-34-2. Further, the percentage peak area for *m*-ethylacetophenone was found to be the highest in the CCD4 19-3-22-4 line compared to the other lines and WT. It is noteworthy that trace amounts of  $\beta$ -ionone were observed in the CCD4 19-3-22-4 profile. The percentage peak area of acetophenone was found to be the lowest in this line.

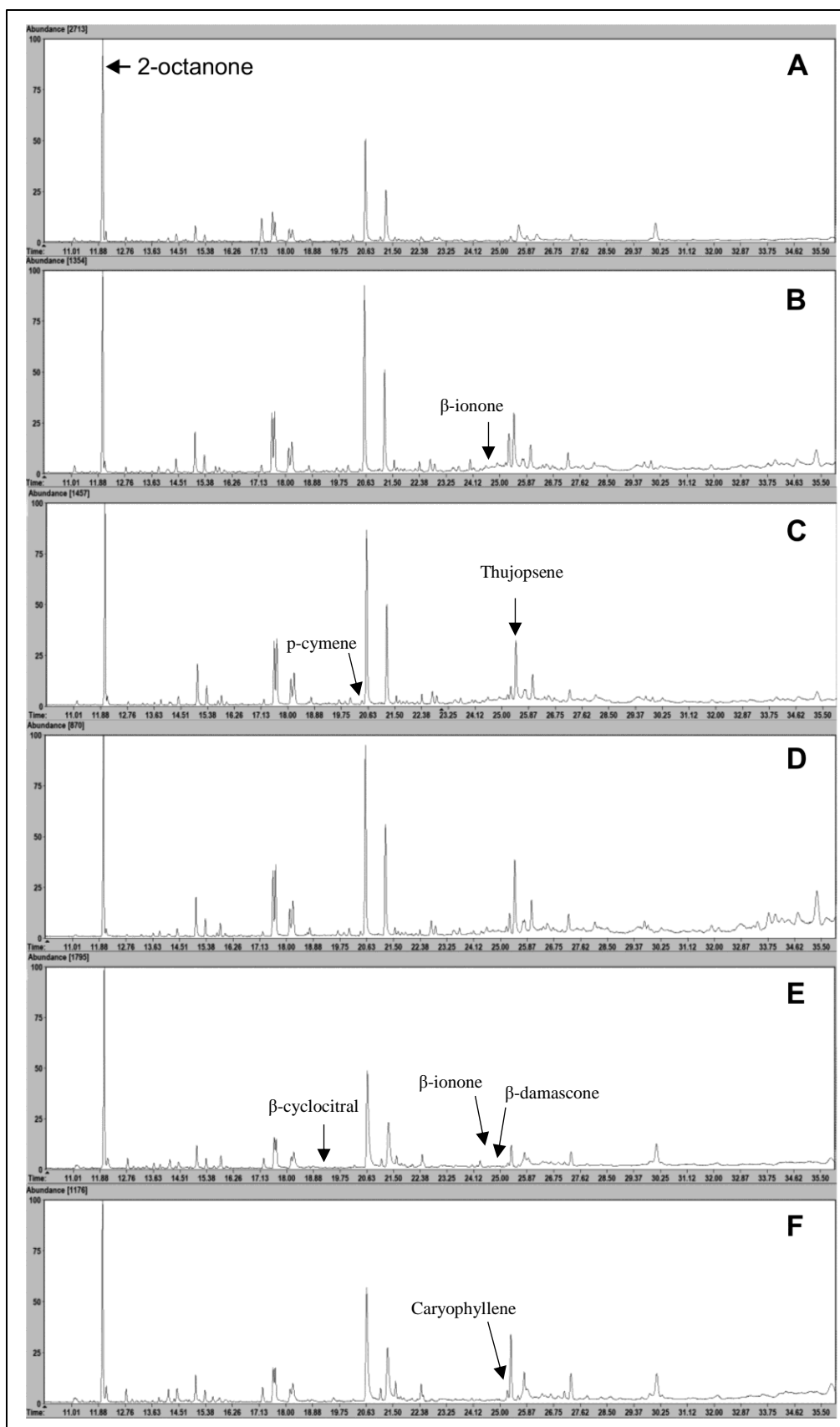
Three volatile apocarotenoids were detected in CCD8 40-1-26-1.  $\beta$ -cyclocitral,  $\beta$ -ionone, and  $\beta$ -damascone were found in notable amounts (4.1 %, 6.6%, and 6.5%, respectively) compared to the other lines and WT. The percentage peak area of benzaldehyde was the lowest in this line.

The percentage peak areas of three sesquiterpenes,  $\alpha$ -longipinene, caryophyllene, and  $\beta$ -chamigrene were found to be the highest relative to the other lines and WT in CCD8 12-3-15-1. The percentage peak area of cinnamaldehyde was found to be the lowest in this line relative to the other lines and WT.

**Table 6: A list of volatile compounds from the NIST spectral database.**

*nd*-not detected; *tr*- traces; each value that represents the % peak area for each compound is an average of at least three biological replicates. RI: Retention index; RT: retention time. Percentage peak area was calculated as a ratio of peak area of each compound to the peak area of the internal standard, 2-octanone.

No.	RT	RI	Compound	Peak area % (average values; % peak areas with respect to internal standard, 2-octanone)					
				WT	CCD4 1-1-9-1	CCD4 17-2-34-2	CCD4 19-3-22-4	CCD8 40-1-26-1	CCD8 12-3-15-1
1	14.431	1029	Acetophenone	6.81	11.75	7.14	5.72	6.27	7.03
2	33.417	1090	3-Thujen-2-ol	<i>nd</i>	<i>nd</i>	<i>nd</i>	11.40	<i>nd</i>	<i>nd</i>
3	25.198	1139	$\alpha$ -Thujenal	<i>nd</i>	<i>nd</i>	<i>tr</i>	4.71	<i>nd</i>	<i>nd</i>
4	20.056	1184	3-Cyclohexen-1-ol	11.58	<i>nd</i>	<i>tr</i>	5.57	<i>nd</i>	<i>nd</i>
5	18.771	1189	Cinnamaldehyde, (E)-	12.25	9.37	7.35	4.93	3.31	2.88
6	25.197	1191	(-)-Myrtenol	<i>nd</i>	<i>nd</i>	3.27	<i>nd</i>	<i>nd</i>	<i>nd</i>
7	17.564	1195	Benzaldehyde	38.80	72.34	20.52	34.02	14.32	23.40
8	19.357	1204	$\beta$ -Cyclocitral	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>	4.12	<i>nd</i>
9	25.252	1209	1-Methylverbenol	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>	3.14	<i>nd</i>
10	22.392	1221	$\alpha$ -Copaene	64.41	33.59	14.98	33.80	<i>nd</i>	<i>nd</i>
11	20.588	1242	m-Ethylacetophenone	11.84	184.38	128.53	203.80	59.39	103.86
12	20.052	1262	p-Cymen-2-ol/p-cymene	<i>nd</i>	<i>nd</i>	12.34	5.72	<i>nd</i>	<i>nd</i>
13	20.054	1284	p-Cymen-7-ol	<i>nd</i>	11.27	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>
14	24.036	1344	$\alpha$ -Cubebene	<i>nd</i>	10.72	<i>tr</i>	<i>tr</i>	<i>nd</i>	<i>tr</i>
15	25.791	1403	$\alpha$ -Longipinene	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>tr</i>	<i>nd</i>	8.65
16	25.739	1416	Thujopsene	9.80	8.08	16.08	10.55	14.50	14.93
17	24.927	1457	3-Buten-2-one/ $\beta$ -ionone	<i>nd</i>	11.66	<i>nd</i>	<i>tr</i>	6.62	<i>nd</i>
18	24.998	1457	$\beta$ -Damascone/3-Buten-1-one	<i>nd</i>	<i>tr</i>	<i>nd</i>	<i>nd</i>	6.54	<i>nd</i>
19	25.741	1458	$\alpha$ -Farnesene	<i>nd</i>	8.68	<i>nd</i>	<i>tr</i>	<i>tr</i>	<i>tr</i>
20	25.197	1490	$\alpha$ -Bulnesene	<i>nd</i>	12.23	<i>nd</i>	<i>tr</i>	<i>nd</i>	<i>nd</i>
21	25.302	1494	Caryophyllene	10.33	20.14	10.04	21.15	22.75	28.68
22	27.105	1507	$\beta$ -Chamigrene	<i>nd</i>	<i>tr</i>	<i>tr</i>	<i>tr</i>	<i>nd</i>	5.35
23	32.882	2072	Cembrene	11.15	<i>nd</i>	<i>nd</i>	<i>tr</i>	<i>nd</i>	<i>nd</i>



Retention time (min)  $\longrightarrow$

**Figure 12: Representative chromatograms of volatile profiles of each Arabidopsis transgenic line and WT.** Volatiles collected by dynamic headspace sampling were analyzed using GC-MS. Each profile represents one line: (A) WT; (B) CCD4 1-1-9-1; (C) CCD4 17-2-34-2; (D) CCD4 19-3-22-4; (E) CCD8 40-1-26-1; (F) CCD8 12-3-15-1. The peak representing the internal standard (2-octanone) is indicated in the WT profile. The peaks representing some of the volatile compounds listed in Table 12 are indicated.

### **3.6 Effect of *CCD4* and *CCD8* overexpression on the feeding damage of *Arabidopsis* leaves by crucifer flea beetles**

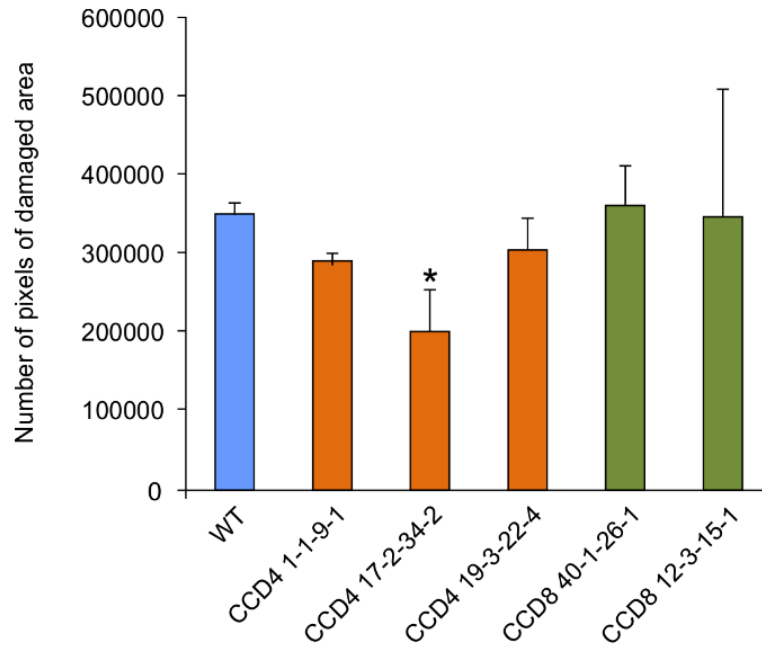
Although overexpression of the *CCD4* and *CCD8* genes had an inconsistent effect on the range and quantity of volatiles of the transgenic lines, it was necessary to study effects on the feeding damage by the crucifer flea beetles. I was interested in this in order to observe any effects these changes in volatile levels may have on the feeding damage of flea beetles.

The results of this analysis showed that there was a pattern of reduced feeding damage by the beetles on the *CCD4* plants compared to *CCD8* and WT plants. In the *CCD4* plants, the total amount of leaf material that was fed on by the beetles was lower than that in the WT plants. On the basis of the analysis of the feeding damage observed in the rosette leaves (Figure 13), it was found that the amount of leaf material that was eaten by the beetles in the *CCD4* 17-2-34-2 lines was significantly lower ( $P \leq 0.05$ ) than the WT lines (Figure 14). The other two *CCD4* lines – *CCD4* 1-1-9-1 and 19-3-22-4 – did not show a significant difference in the feeding damage compared to WT; nevertheless, there appeared to be a trend in these lines, which indicated decreased feeding damage than that in WT. In the case of the two *CCD8* lines, it appeared from the pattern of feeding damage that there were no noticeable differences between them and the WT lines.



**Figure 13: Analysis of feeding damage of six-week-old Arabidopsis plants.**

Crucifer flea beetle feeding damage was assessed using Corel Photo Paint 12. The area of damage for each leaf was quantified using the Corel Photo Paint 12 software. The images are representative of all the damaged leaves of four (A) CCD4 and (B) WT Arabidopsis plants. Quantification was performed in three trials for each line including the WT. Each trial consisted of four six-week-old Arabidopsis plants enclosed in a chamber with 200 flea beetles, and the feeding trial was performed over a 24 h period.



**Figure 14: Quantification of feeding damage by the crucifer flea beetles.** The damage to the leaves of Arabidopsis plants by the flea beetles was quantified in each line. Each bar represents the mean of at least three biological replicates. The error bars represent the standard error of mean. The means of the WT and each transgenic line were compared using two-tailed T-test. The level of significance indicated by “\*” represents  $P \leq 0.05$ .



## Chapter 4: Discussion

### 4.1 Overview

Plant-insect interactions may be mutual or parasitic in nature, and one of the many complex mechanisms plants have evolved to help them cope with damage inflicted by insects is the release of volatile organic compounds (Pare and Tumlinson, 1999). These volatile substances are secondary metabolites released from the plant and are involved in enhancing plant defenses and improving scent and aroma quality in flowers and fruits (Dudareva and Pichersky, 2008). The volatile compounds are represented by various classes of compounds, such as terpenoids, phenylpropanoids/benzoids, fatty acid derivatives, and amino acid derivatives, to name a few (Dudareva and Pichersky, 2000). Among these major classes, terpenoids are known to be present in great variety (Dudareva et al., 2004). The diverse class of terpenoids includes a group of compounds called apocarotenoids, which are derived from the oxidative cleavage of carotenoids (Wahlberg and Eklund, 1998). The carotenoid catabolism pathway produces a wide range of useful apocarotenoids, which include phytohormones, such as ABA and strigolactone, and flavor/aroma compounds, such as  $\beta$ -ionone,  $\beta$ -damascone,  $\alpha$ -ionone,  $\beta$ -cyclocitral,  $\beta$ -damascenone, theaspirone etc. (Simkin et al., 2004a; Simkin et al., 2004b). The CCD and NCED enzymes associated with the production of apocarotenoids are commonly found in many plants and bacteria. In *Arabidopsis* this family consists of nine genes – four *CCD* genes (*CCD1*, *CCD4*, *CCD7*, and *CCD8*) and five *NCED* genes (*NCED2*, *NCED3*, *NCED5*, *NCED6*, and *NCED9*). Each of the enzymes encoded by these genes is presumed to have its own substrate of preference. An interesting study by Wei et al.

(2011) focused on characterization of *CCD1* by studying the effects of overexpressing the gene in Arabidopsis. From the study by Wei et al. (2011), *CCD1* overexpression in Arabidopsis produced enhanced levels of  $\beta$ -ionone. Further, the study concluded that  $\beta$ -carotene was the substrate being cleaved, thus resulting in the generation of  $\beta$ -ionone.  $\beta$ -Ionone is known to impart fruit flavor and flower fragrance (Schwartz et al., 2001; Wei et al., 2011). In addition, it plays a role in insect deterrence (Wei et al., 2011). Based on feeding assays, it was evident that the enhanced levels of  $\beta$ -ionone in transgenic plants significantly deterred feeding damage by the crucifer flea beetles. Taking into account the study by Wei et al. (2011), I was interested in characterizing the additional members of the CCD family in Arabidopsis; in addition to the characterization as performed on *CCD1* in the previous study, I was interested in the determination of the carotenoid content and volatile profiles of the overexpression lines. The ultimate goal was to investigate the effects of overexpressing the other *CCD* genes on feeding by crucifer flea beetles.

## **4.2 Accumulation of carotenoids in the leaves of *CCD4* and *CCD8* overexpressors**

Since the *CCD* genes are involved in the oxidative cleavage of carotenoids, it was necessary to analyze the carotenoid content in the leaves of the *CCD* overexpression lines. Levels of the three most abundant carotenoids in Arabidopsis leaves – lutein,  $\beta$ -carotene, and violaxanthin – were determined. A previous study by Ohmiya et al. (2006) studied the effects of suppression of *CmCCD4a* (*Chrysanthemum morifolium* *CCD4*) on carotenoid accumulation, using a RNA interference (RNAi) approach (Ohmiya et al., 2006). Based on organ-specific expression analysis of *CmCCD4* in the ornamental plant,

Chrysanthemum, *CmCCD4a* expression was limited to the flower petals and was not found to be present in significant levels in other parts of the plant. In order to determine the role of *CmCCD4a* in the formation of petal color, transgenic lines with reduced expression of *CmCCD4* were studied. This study revealed that there was a clear relationship between the carotenoid content and the *CmCCD4a* mRNA abundance (Ohmiya et al., 2006). The suppression of *CmCCD4a* expression affected the petal color of the chrysanthemums, as the *CmCCD4a* RNAi lines exhibited yellow petals suggesting that *CmCCD4a* plays a role in the white color formation in the petals. This indicated that the *CmCCD4a* suppression had a direct association with the carotenoid content of the plant. Although biochemical and enzymatic characterization of the Arabidopsis CCD4 has not been performed, the deduced amino acid sequence of *CmCCD4a* showed highest homology with that of *CCD4* among the members of the Arabidopsis CCD family (61% homology; (Ohmiya et al., 2006). Further, *CmCCD4a* shares a feature with the Arabidopsis CCD4 wherein both enzymes contain a plastid-targeting transit peptide at the N terminus and four highly conserved His residues that may be involved in coordinating a non-haeme iron required for enzymatic activity (Schwartz et al., 1997b; Tan et al., 1997). In addition, Campbell et al. (2010) showed that silencing of *StCCD4* in potato (*Solanum tuberosum*) enhanced the total carotenoid content of the tubers. HPLC analysis revealed that the main change in the carotenoid content was a significant increase in violaxanthin (Campbell et al., 2010), suggesting that violaxanthin is cleaved by *StCCD4*. As in the case of *StCCD4* study in potato tubers, silencing of *StCCD8* using RNAi was also performed in potato. The developing tubers in the *StCCD8*-RNAi lines showed that the line with the lowest *StCCD8* expression had up to a 5.6-fold increase in the total tuber

carotenoid content as compared to the WT control. However, studies on silencing of *CCD8* expression in tomato (Vogel et al., 2010) and kiwi fruit (Ledger et al., 2010) did not report any changes in total carotenoid levels as observed in the developing tubers of *StCCD8*-RNAi potato plants. Since the levels of carotenoids were not analyzed in the study by Wei et al. (2011) on *CCD1* characterization, it is not known if the overexpression of *CCD1* impacted the carotenoid levels. However, the results of my research were very interesting and contrary to the results from the previous studies on *CCD4* and *CCD8* (Figure 8 A, B & C and Figure 9 A & B). Overexpression of *CCD4* and *CCD8* in *Arabidopsis* resulted in enhanced accumulation of lutein, violaxanthin, and  $\beta$ -carotene. This was quite contrary to the idea that overexpression of *CCD4* and *CCD8* would result in a higher rate of carotenoid degradation, and thus reduced carotenoid accumulation. I hypothesize that this accumulation of carotenoids may be due to a positive feedback regulation in the leaves as a result of enhanced carotenoid degradation in order to maintain a balance between levels of carotenoids and chlorophyll that is necessary for the integrity of the photosynthesis system (Ruiz-Sola and Rodriguez-Concepcion, 2012). Alternatively, this accumulation may be due to the fact that the overexpression of *CCD4* and *CCD8* does not influence the activity of CCDs. Another striking point in these results is that the relative levels of carotenoid accumulation in each line in both *CCD4* and *CCD8* appear to be proportional to the relative transcript levels of the *CCD4* and *CCD8* genes, respectively (Figure 6 and 7). The *CCD4* and *CCD8* lines with highest transcript levels (*CCD4* 1-1-9-1 and *CCD8* 12-3-15-1, respectively) showed a significant accumulation of all three carotenoids. Two of the *CCD4* lines (*CCD4* 17-2-34-2 and 19-3-22-4), which showed a lower *CCD4* transcript level compared to *CCD4* 1-

1-9-1, did not show a significant increase in the carotenoid levels compared to WT; however, there was an evident trend in the accumulation of carotenoids in these lines (Figure 8 B & C). A previous study by Lindgren et al. (2003) showed that overexpression of endogenous phytoene synthase resulted in plants with increased levels of  $\beta$ -carotene, lutein, and violaxanthin (Lindgren et al., 2003). My results suggest that the overexpression of *CCD4* and *CCD8* could possibly result in the activation of phytoene synthase (PSY), which catalyzes a rate-limiting step in the carotenoid biosynthesis pathway (Figure 1), and possibly other carotenoid biosynthesis enzymes (Shewmaker et al., 1999; Yu et al., 2008). This was further investigated by analyzing expression of key genes in carotenoid biosynthesis in *CCD4* and *CCD8* overexpression lines.

### **4.3 Carotenoid accumulation in overexpression lines correlates with expression of carotenoid biosynthesis genes**

In order to investigate the effect of overexpressing *CCD4* and *CCD8* genes on the accumulation of carotenoids in the plants, several genes from the carotenoid biosynthesis pathway were analyzed by qRT-PCR as described in Yu et al. (2008). The transcript level of *PSY* was significantly higher in the *CCD4* overexpression lines as compared to WT. However, the overexpression of *CCD4* did not affect the level of *PDS*, which encodes for PDS, the enzyme subsequent to PSY in the pathway that acts in tandem with  $\zeta$ -carotene isomerase on phytoene to produce  $\zeta$ -carotene; nevertheless, an increasing trend in *PDS* expression levels was observed compared to the WT control, although the increase was not statistically significant. An increase in the transcript levels of *PSY* could cause the accumulation of the carotenoids (lutein,  $\beta$ -carotene, and violaxanthin) in the *CCD* overexpression lines (Figure 10 A, B & C). In the case of the *CCD8* lines, *CCD8* 40-1-

26-1 exhibited the same pattern as that in the CCD4 lines, wherein a significant increase in the transcript levels of *PSY* was observed in this line compared to the WT; however, this was inconsistent with the other CCD8 line CCD8 12-3-15-1, which showed a significant decrease in the transcript levels compared to the WT plants. This implies that the higher transcript level of *CCD8* in CCD8 12-3-15-1 inversely affected the levels of *PSY* in this line. However, although there was a consistent accumulation of lutein in both the CCD8 lines, the inconsistency in the expression levels of *PSY* remains unclear. Further, there was a decrease in the transcript level of *PDS* in both CCD8 lines compared to WT (Figure 11). Analysis of more *CCD8* overexpression lines would be necessary to fully understand the role *CCD8* overexpression plays in regulating carotenoid biosynthesis.

Another remarkable finding from the qRT-PCR analysis of the carotenoid genes was the transcript level of lycopene  $\beta$ -cyclase (*bLYC*) in the *CCD4* lines, which was significantly lower relative to the WT (Figure 10 A). *bLYC* is involved in the cyclization of lycopene in the two branch points - in one branch point two lycopene  $\beta$ -cyclases catalyse the production of  $\beta$ -carotene, and in the other branch point lycopene  $\beta$ -cyclase and lycopene  $\epsilon$ -cyclase coordinately produce  $\alpha$ -carotene, which is then hydroxylated to lutein (Figure 1). It could be concluded from these data that although the overexpression of *CCD4* resulted in a decrease in the transcript levels of *bLYC* in all the three *CCD4* lines, there is a likelihood that there is a compensation in the transcript levels of *LUT1*, which was observed by the significant increase in the *LUT1* transcript levels in two *CCD4* lines (CCD4 1-1-9-1 and 17-2-34-2; Figure 10 B). *LUT1* plays a role in the hydroxylation of the  $\epsilon$ -ring of  $\beta$ ,  $\epsilon$ -carotenoids, e.g.,  $\alpha$ -carotene (Kim and DellaPenna,

2006). Further, the study by Kim and Penna (2006) showed that *lut1* mutants had significantly lower levels of lutein compared to the WT plants. The higher levels of *LUT1* in CCD4 1-1-9-1 and 17-2-34-2 compared to WT in my results suggest a possible link between the *LUT1* transcript levels and lutein accumulation in all *CCD4* lines. However, this would have to be investigated further due to the inconsistency in the *LUT1* expression levels among the *CCD4* lines.

The transcript levels of *VDE* in CCD4 19-3-22-4 was significantly lower than in WT; this line happened to display significantly low *CCD4* transcript levels compared to WT. Since *VDE* is involved in a reversible reaction in the pathway wherein violaxanthin is converted to zeaxanthin, it could be expected that the levels of violaxanthin in this line would be higher compared to WT; however although violaxanthin showed an accumulating trend in this line, this was not significant compared to WT. One of the other *CCD4* lines 17-2-34-2 showed a significant increase in the levels of *VDE* compared to WT, and the levels of violaxanthin accumulation were not different from WT. With respect to CCD4 1-1-9-1, it was surprising that the expression level of *VDE* was not significantly different from WT considering the relatively high level of violaxanthin in this line (Figure 10 C).

Another interesting finding was the significant increase in *ABA2* transcript level in only one of the *CCD4* lines - CCD4 19-3-22-4 (Figure 10 C) compared to WT. Studies on *aba2* mutants (Schwartz et al., 1997a) pointed out that *aba2* mutants were unable to convert xanthoxin to ABA. Although ABA analysis was not performed in this study, it would be critical to conduct this analysis to fully understand the effect of *CCD4* overexpression on ABA accumulation.

#### 4.4 Overexpression of *CCD* genes causes changes in volatile profiles

To investigate the effects of the *CCD4* and *CCD8* overexpression on volatile emissions, analysis of the volatiles collected from the headspace of *CCD4* and *CCD8* transgenic plants was performed using GC-MS. The results of these experiments showed that there were differences in the range and level of volatiles in the transgenic lines relative to WT control (Figure 12).

Table 6 presents a list of volatile compounds that were collected from the headspace of the transgenic and WT plants. Among the 23 compounds, only three compounds were volatile apocarotenoids ( $\beta$ -ionone,  $\beta$ -damascone, and  $\beta$ -cyclocitral) while the rest belonged to the class of monoterpenes, sesquiterpenes, and benzenoids. The volatiles in the line that showed highest *CCD4* overexpression compared to WT – *CCD4* 1-1-9-1 had the highest total amount of volatiles compared to the other *CCD4* lines, *CCD8* lines, and WT. This is consistent with the high level of *CCD4* gene expression in this line. The percentage peak area of  $\beta$ -ionone in *CCD4* 1-1-9-1 was found to be the highest compared to the other lines, and this compound was undetectable in WT. This is supported by findings of a study by Huang et al. (2009b) in which the functions of recombinant *E. coli*-produced *CCD4* genes of apple (*MdCCD4*), Chrysanthemum (*CmCCD4a*), rose (*RdCCD4*), and osmanthus (*OfCCD4*) and Arabidopsis *CCD4* (*AtCCD4*) were investigated. The authors found that *CmCCD4* and *MdCCD4* cleaved  $\beta$ -carotene at the 9, 10 (9', 10') double bond positions to yield  $\beta$ -ionone, while *OfCCD4*, *RdCCD4*, and *AtCCD4* were inactive towards these substrates. Instead, *in vitro* assays showed that *AtCCD4* and *RdCCD4* could cleave the C<sub>30</sub>-apocarotenoid 8'-apo- $\beta$ -caroten-8'-al to  $\beta$ -ionone (Huang et al., 2009b). However *in*



*planta* activity of AtCCD4 has not yet been elucidated. Although CCD1 and CCD4 enzymes cleave carotenoids at the same positions (9, 10 and 9', 10'), CCD4 enzymes are more specific regarding their substrate unlike CCD1, which has a broad substrate tolerance and produces numerous C<sub>13</sub> products (Huang et al., 2009a; Schwartz et al., 2001; Simkin et al., 2004a). Studies by Huang et al. (2009) concluded that CCD4 could not cleave linear carotenoids such as lycopene and cis- $\zeta$ -carotene, or carotenoids containing a hydroxyl group such as zeaxanthin and lutein. Further, it was suggested that CCD4 only cleaves cyclic non-polar carotenoids such as  $\beta$ -carotene (Huang et al., 2009b).

The results from previous studies on the products of CCD4 function correlate with my findings, wherein the line with highest *CCD4* expression (CCD4 1-1-9-1) showed the highest level of  $\beta$ -ionone emission, another line, CCD4 19-3-22-4 had only traces of  $\beta$ -ionone, and no  $\beta$ -ionone was detected in the CCD4 17-2-34-2 profile (Table 6). Apart from the presence of volatile apocarotenoids in the CCD4 lines, I also detected some other volatiles, especially benzaldehyde, which is associated with plant insect interactions. This volatile compound had the highest percentage peak area in CCD4 1-1-9-1 compared to the other lines. Benzaldehyde plays a role in synergistic association between plants and insects (Reddy and Guerrero, 2004). Another volatile compound, acetophenone, showed the highest peak area in CCD4 1-1-9-1. Acetophenone is an aromatic compound known to be responsible for scents in flowers, and is a component of flavors and fragrances in honey, plums and strawberries (Hilton and Cain, 1990), and is an attractant to insects (Buttery et al., 1984). Three sesquiterpenes were also found to have the highest peak areas in CCD4 1-1-9-1. These are  $\alpha$ -farnesene,  $\alpha$ -bulnesene, and  $\alpha$ -

cubabene, which are known to be emitted exclusively from flowers of *Arabidopsis* (Chen et al., 2003). Overall, the CCD4 lines showed a blend of a wide range of volatiles that are involved in insect deterrence as well as in pollination.

A striking point in the case of the CCD4 17-2-34-2 line was the high levels of *p*-cymene. This was also observed in CCD4 19-3-22-4, but at lower levels than CCD4 17-2-34-2. A previous study by Bleeker et al. (2009) showed that *p*-cymene, a monoterpene, in tomato has a strong repelling effect on whiteflies (Bleeker et al., 2009).

The high level of expression of *ABA2* in the CCD4 19-3-22-4 line suggests the possibility of high ABA levels in this line. ABA is known to play a major role in controlling seed germination and plant responses during abiotic stress responses caused by drought, salt and wounding (Christmann et al., 2006). Previous studies on ABA suggested that levels of ABA in maize increased during attack by the specialist root herbivore, the western corn rootworm (*Diabrotica virgifera virgifera*; (Erb et al., 2009). Further, ABA was recently implicated in the regulation of defense-related genes (Anderson et al., 2004; Lorenzo et al., 2004). However, to date, there have been no studies that implicated ABA in insect resistance in plants.

Of all the CCD8 lines, CCD8 40-1-26-1 showed the presence of three volatile apocarotenoids –  $\beta$ -ionone,  $\beta$ -damascone, and  $\beta$ -cyclocitral. *In vitro* studies have shown that CCD7 cleaves  $\beta$ -carotene at the 9, 10 (9', 10') double bond positions generating the C<sub>27</sub> compound  $\beta$ -apo-10'-carotenal and the C<sub>13</sub> compound  $\beta$ -ionone. When CCD7 was co-expressed in *E. coli* with CCD8,  $\beta$ -apo-13-carotenal was produced by secondary cleavage of the  $\beta$ -apo-10'-carotenal (formed by CCD7) at the 13, 14 (13', 14') double bond

position (Schwartz et al., 2004). This suggests that CCD7 and CCD8 act in a sequential manner to produce SL. On the basis of the previous studies, it would be predicted that since CCD7 and CCD8 act in a concerted manner, the overexpression of *CCD8* would not affect levels of volatile apocarotenoids that are by-products of SL biosynthesis. Although  $\beta$ -damascone and  $\beta$ -cyclocitral are known in fruit flavor and aroma (Pinho et al., 2013; Schmidt et al., 2013), their role in insect resistance has not been documented.

#### **4.5 Overexpression of *CCD4* deters feeding by the crucifer flea beetle**

The results from the insect feeding bioassay indicated that the CCD4 lines, especially CCD4 17-2-34-2, had a deterring effect on the crucifer flea beetle compared to the CCD8 lines (Figure 14). CCD4 17-2-34-2 showed significantly decreased levels of feeding damage compared to the WT. It was observed that CCD4 17-2-34-2 plants had enhanced levels of *p*-cymene, which was reported to have a repelling effect on whiteflies feeding on tomato plants (Bleeker et al., 2009). CCD4 17-2-34-2 plants had the lowest levels of  $\alpha$ -copaene compared to the other lines, including WT.  $\alpha$ -Copaene is a sesquiterpene that was previously shown to enhance the mating success of the male flies of the Mediterranean fruit fly (*Ceratitis capitata*) when exposed to fruits containing this volatile compound (Shelly, 2005). Although CCD4 1-1-9-1 had the highest percentage peak area of  $\beta$ -ionone, there were no significant differences in the feeding damage observed in this line relative to WT; however, the overall trend in the feeding damage showed that the CCD4 lines deterred insect feeding compared to the CCD8 lines. With respect to the CCD8 lines, although there was a presence of three volatile apocarotenoids, namely  $\beta$ -ionone,  $\beta$ -damascone and  $\beta$ -cyclocitral, there was no feeding deterrence that was observed in these lines. This could indicate that these volatiles at the levels in which

they are produced may not have an effect on insect feeding. There were no statistical differences between the CCD8 lines and WT in feeding damage by flea beetles (Figure 14).

#### **4.6 Correlation between carotenoid accumulation and production of volatile apocarotenoids and insect feeding damage**

This study showed that there was an accumulation of key carotenoids, namely lutein,  $\beta$ -carotene, and violaxanthin, in the leaves of CCD4 and CCD8 overexpression lines, even though a drop in the levels of these compounds had been expected due to a predicted increase in carotenoid catabolism. This accumulation could possibly be due to several reasons: 1) positive feedback regulation in the carotenoid biosynthesis pathway in the overexpression lines; 2) possibility that the overexpression of the *CCD* genes does not affect the activity of CCD enzymes in the plants; 3) the overexpression lines may be under stress due to the overexpression of the *CCD* genes. Interestingly, the transcript level of the *PSY* gene encoding the phytoene synthase that catalyzes a rate-limiting step in the carotenoid biosynthesis pathway was significantly higher in all the three CCD4 lines compared to WT; this was reflected in the increased accumulation of carotenoids in the CCD4 lines compared to WT. However, although there was an accumulation of carotenoids in the CCD8 lines, the pattern of *PSY* gene regulation was not consistent with those of the CCD4 lines. Only CCD8 40-1-26-1 showed an increase in *PSY* transcript levels relative to WT, whereas the other line (CCD8 12-3-15-1) showed a significant decrease in *PSY* transcript level.

The significant increase in transcript levels of *PSY* observed in the CCD4 line and one CCD8 line may be correlated to the volatile apocarotenoids that were observed in these. The CCD4 line showing the highest level of *CCD4* expression (CCD4 1-1-9-1) had the greatest abundance of  $\beta$ -ionone (exhibited a percentage peak area of 11.7 %) compared to the other lines and WT (Table 6). However, the levels of volatile apocarotenoids were not necessarily reflected in feeding damage that was observed by the crucifer flea beetles. The CCD4 line that showed the presence of the highest levels of *p*-cymene, exhibited significantly higher levels of feeding deterrence against crucifer flea beetle feeding. On the other hand, insect feeding was not affected in CCD4 lines showing the presence of the volatile apocarotenoids such  $\beta$ -ionone,  $\beta$ -damascone, and  $\beta$ -cyclocitral. This may be due to the fact that the levels of these apocarotenoids may not be high enough to repel the flea beetles effectively. Alternatively, this could imply that not all the volatile apocarotenoids are associated with insect feeding deterrence. Instead, some of the other volatiles (non apocarotenoids) may be the cause for the insect deterrence.

## Chapter 5: Perspectives and Future Work

The concept of this research was based on the initial results of a study by Wei et al. (2011) on the effects of overexpressing *CCD1* in *Arabidopsis* on the plant volatile profile and deterrence of flea beetle herbivory. Thus, my goal was to examine the effect of overexpression of other *CCD* and *NCED* genes on the aforementioned traits in *Arabidopsis*.

Constitutive overexpression of *CCD4* and *CCD8* in *Arabidopsis* was assessed for its effect on carotenoid accumulation in the leaves and the volatile profiles of the whole plants. My analysis suggested there was an accumulation of carotenoids in leaves of *CCD4* and *CCD8* overexpression lines compared to WT. This is contrary to studies that showed that silencing of *CCD4* and *CCD8* genes led to an accumulation of carotenoids. It would be interesting to quantify the level of ABA in the line that showed a high level of *ABA2* gene expression in order to determine if the *ABA2* expression will affect the levels of ABA in the plants. Further, it would be critical to assess the chlorophyll content of the overexpression lines in order to establish the ratio of chlorophyll to carotenoids. Moreover, unlike the effect of *CCD1* overexpression on volatile emissions and insect feeding (Wei et al., 2011), there were no particular and consistent effects of *CCD4* and *CCD8* overexpression on the volatile profiles of the plants. Nevertheless, some lines showed the presence of other monoterpenes, such as *p*-cymene, which although related to carotenoid precursors, are not catabolites of the carotenoid pathway. These monoterpenes are known to have repelling effects on insects (Bleeker et al., 2009). A follow up study to this would be to perform volatile collection while the crucifer flea beetles feed on the

Arabidopsis plants. This would help identify any specific volatiles involved in defense against herbivore attack by this beetle. It would be necessary to identify specific volatile compounds and perform experiments using various concentrations of volatile standards to identify the concentration that would have the greatest impact on insects.

It would also be informative to investigate the effect of co-overexpressing two or more *CCD* and *NCED* genes. Although it is quite clear that the mechanism of these enzymes are quite complex, it would be useful to identify the biochemical functions of these enzymes *in vivo*, since some of these enzymes work in a coordinated manner, such as the role that CCD7 and CCD8 enzymes play in SL biosynthesis (Schwartz et al., 2004).

The *CCD1* gene, which was previously studied by Wei et al. (2011), along with two tomato CCD1 (*LeCCD1a* and *LeCCD1b*), have been transformed into the microtom tomato (*Solanum lycopersicum*). Since the effect of overexpressing the *CCD1* gene on volatile emissions and insect feeding was established in the Arabidopsis model system, it would be worth investigating the effects in an actual economically important crop; e.g. tomato.

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# CURRICULUM VITAE

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## **Education**

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- The University of Western Ontario  
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- University of Mumbai  
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2005 – 2007, M.Sc. Biophysics
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## **Awards and Scholarships**

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2006-2007	Awarded scholarship for being among the top three students in the M.Sc. Biophysics program, University of Mumbai, Mumbai, India

## **Work Experience**

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2011 – 2013	Graduate Teaching Assistant, University of Western Ontario, London, Canada
2009 – 2011	Research assistant/Application Specialist, SafLabs Pvt. Ltd., Mumbai, India
2009	Research assistant, Dr. Bhiwgade's Biotechnology Research Centre, Mumbai, India

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**Lakshminarayan, S.,** Wei, S., Gruber, M.Y., Bernards, M.A., Qubbaj, T., and Hannoufa, A. (2013). "Role of carotenoid cleavage dioxygenases in volatile emissions and insect resistance in *Arabidopsis*", Canadian Society of Plant Biologists (CSPB) Eastern Regional Meeting, University of Toronto, Mississauga, ON, Canada.

**Lakshminarayan, S.,** Wei, S., Gruber, M.Y., Bernards, M.A., Tian, L.N., Qubbaj, T., and Hannoufa, A. (2012). "Role of carotenoid cleavage dioxygenases in volatile emissions and insect resistance in *Arabidopsis*", 3rd Annual Biology Graduate Research Forum 2012, University of Western Ontario, London, ON, Canada.

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